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EGFR are overexpressed in the majority of ER-negative, hormone-resistant and poor prognosis breast cancers. Our goal is to exploit EGFR overexpression to selectively target the Auger electron-emitting radiopharmaceutical, ^{111}In-hEGF to breast cancer cells for treatment of the disease. ^{111}In-hEGF was highly radiotoxic *in vitro* to MDA-MB-468 breast cancer cells overexpressing EGFR (1-2 X 10^6 receptors/cell) but not to MCF-7 breast cancer cells with a 100-fold lower level of EGFR. ^{111}In-hEGF was >100-400 fold more cytotoxic to MDA-MB-468 cells than chemotherapeutic agents (IC_{50} 70 pM vs. 6-30 nM) and low concentrations (70 pM) of ^{111}In-hEGF produced the same growth inhibition as 4 Gy of γ-radiation. The cytotoxicity was amplified (IC_{50} 15 pM) by conjugation of hEGF with HSA and derivatization with multiple DTPA metal chelators for ^{111}In. Mice implanted with MDA-MB-468 tumors treated with 5 doses of 500 μCi of ^{111}In-hEGF exhibited tumor growth arrest. There was no evidence of normal tissue toxicity as measured by changes in body weight, histopathological examination of liver and kidneys and serum ALT and Cr levels. There was a slight but not significant decrease in WBC and platelets. Our results are highly promising for the development of ^{111}In-hEGF as a novel treatment for breast cancer.

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INTRODUCTION

The epidermal growth factor receptor (EGFR) is a transmembrane receptor tyrosine kinase which specifically binds human epidermal growth factor (hEGF) and is overexpressed in the majority (>90%) of estrogen receptor-negative, hormone-resistant and poor prognosis breast cancers (1). The level of EGFR overexpression in breast cancer may reach 10^6 receptors/cell, more than 100-fold higher than on normal epithelial cells (<10^4 EGFR/cell). Binding of hEGF to the receptor activates an intracellular signaling pathway which ultimately results in upregulation of gene expression and cell division. hEGF is rapidly internalized into the cytoplasm following binding to its cell surface receptor, and a proportion of molecules are translocated to the cell nucleus (2). In this research project, it is hypothesized that the internalization and nuclear translocation of hEGF can be exploited to selectively insert the Auger electron-emitting radionuclide, indium-111 (^{111}In) into the cytoplasm and nucleus of breast cancer cells overexpressing EGFR, where the short-range electrons are highly damaging to DNA, resulting in cell death. The subcellular range of the Auger electrons should limit the radiotoxicity of the radiopharmaceutical to breast cancer cells which overexpress EGFR and are able to internalize sufficient quantities of the radionuclides. Since <3% of bone marrow stem cells express EGFR (3), the radiopharmaceutical in theory should not be toxic to the bone marrow, but may be toxic to normal tissues such as the liver and kidneys which express moderate levels of EGFR (~10^5 receptors/cell) (4,5). An objective of the research project is to evaluate the cytotoxicity of ^{111}In-hEGF and a novel ^{111}In-human serum albumin-hEGF bioconjugate (^{111}In-HSA-hEGF) against human breast cancer cells *in vitro* in comparison with conventional treatments for breast cancer such as chemotherapy and external γ-radiation. A second objective is to evaluate the anti-tumour effects and normal tissue toxicity of ^{111}In-hEGF (or ^{111}In-HSA-hEGF) against human breast cancer xenografts implanted in athymic mice *in vivo*. The rationale for using the ^{111}In-HSA-hEGF bioconjugate was to increase the molecular size of hEGF to slow its blood clearance and promote tumour accumulation as well as provide sites for conjugation of multiple diethylenetriaminepentaacetic acid (DTPA) metal chelators for ^{111}In to increase the specific activity of the radiopharmaceutical and thereby maximize the delivery of radioactivity to the tumours.

BRIEF SUMMARY OF RESEARCH ACCOMPLISHED IN YEAR 1 (1998-1999)

The following represents a brief summary of the research accomplished in the 1st year of the project. Please consult the *August 1999 Annual Report* for a detailed description.

Task 1: Construction, expression, purification and testing of a novel hEGF-C_H1 fusion protein.

In the 1st year of the project, a recombinant hEGF-C_H1 fusion protein was constructed, expressed in *E. coli* and tested for purity and receptor binding against MDA-MB-468 human breast cancer cells overexpressing EGFR (6) . The protein was successfully produced but only small quantities of correctly folded protein could be isolated and the protein was relatively insoluble which did not allow concentration which was required for DTPA derivatization and labeling with ^{111}In. The research strategy was therefore changed to construct instead a human serum albumin (HSA)-hEGF bioconjugate which had properties similar to those of the hEGF-C_H1 fusion protein (ie. higher molecular weight and multiple sites for DTPA derivatization) but was much more soluble.

BRIEF SUMMARY OF RESEARCH ACCOMPLISHED IN YEAR 2 (1999-2000)

The following represents a brief summary of the research accomplished in the 2nd year of the project. Please consult the *August 2000 Annual Report* for a detailed description.

Task 2: Construction and purification of a novel human serum albumin (HSA)-hEGF bioconjugate

A novel HSA-hEGF bioconjugate was successfully constructed by chemically linking thiolated HSA with maleimide-derivatized hEGF. The HSA-hEGF bioconjugate was purified by ultrafiltration yielding a relatively homogeneous protein with M_r 62-67 kDa by SDS-PAGE and size-exclusion HPLC which was positive for HSA and hEGF by Western blot. The bioconjugate was soluble in aqueous buffers and was easily concentrated to 5-10 mg/mL for derivatization with multiple DTPA metal chelators for labeling to high specific activity with ^{111}In.

Task 3: Radiolabeling of HSA-hEGF with ^{111}In and testing for receptor binding, internalization and nuclear translocation in EGFR-overexpressing breast cancer cells

HSA-hEGF retained its receptor-binding properties *in vitro* against MDA-MB-468 breast cancer cells, but the affinity constant (K_a) was reduced about 15-35 fold compared to hEGF. HSA-hEGF was derivatized with multiple DTPA metal chelators (as many as 23 per molecule) preferentially onto the HSA domain which significantly increased the specific activity when labeled with ^{111}In compared to hEGF which could only be derivatized with a maximum of 1-2 DTPA metal chelators per molecule (7). Analagous to hEGF, HSA-hEGF was rapidly internalized into the cytoplasm and translocated to the cell nucleus in MDA-MB-468 breast cancer cells as assessed by fluorescence microscopy using fluorescein-derivatized bioconjugate and cell fractionation experiments using ^{111}In-labeled bioconjugate.

Task 4: In vitro testing of cytotoxicity of ^{111}In-hEGF, chemotherapy and external γ-radiation against EGFR-overexpressing human breast cancer cells.

The relative antiproliferative activity of ^{111}In-hEGF against MDA-MB-468 breast cancer cells *in vitro* was compared with that of chemotherapeutic agents or external γ-radiation delivered by a ^{137}Cs source. These experiments demonstrated that ^{111}In-hEGF was at least 100-500 fold more cytotoxic (on a molar concentration basis) against EGFR-overexpressing breast cancer cells than the chemotherapeutic agents paclitaxel, doxorubicin, methotrexate or camptothecin and several logarithms more potent than 5-fluorouracil. The 50% cell growth inhibitory concentration (IC_{50}) was <70 pM for ^{111}In-hEGF but was 6 nM for paclitaxel, 15-30 nM for doxorubicin, camptothecin and methotrexate and 4 μM for 5-fluorouracil. ^{111}In-hEGF (70 pM) produced the same growth-inhibitory effect as 4 Gy of high dose rate external γ-radiation.

Task 5: Comparison of the biodistribution and pharmacokinetics of ^{111}In-HSA-hEGF and ^{111}In-hEGF in mice implanted with human breast cancer xenografts.

The biodistribution of ^{111}In-HSA-hEGF was compared with that for ^{111}In-hEGF in athymic mice implanted with subcutaneous MDA-MB-468 breast cancer xenografts. The tumour accumulation

was not significantly increased for ^{111}In-HSA-hEGF compared to ^{111}In-hEGF (1.42 ± 0.37 % i.d./g vs. 1.17 ± 0.28 % i.d./g respectively) and tumour/blood ratios were lower (6.9 ± 1.9 vs. 10.3 ± 2.2 respectively) due to a prolonged retention of ^{111}In-HSA-hEGF in the blood compared to ^{111}In-hEGF (0.25 ± 0.02% i.d./g vs. 0.11 ± 0.02% i.d./g). Although the tumour accumulation of ^{111}In-HSA-hEGF was not improved compared to ^{111}In-hEGF, ^{111}In-HSA-hEGF may deliver more radioactivity to the breast cancer cells *in vivo* due to a much higher specific activity when labeled with ^{111}In, as a result of higher substitution with DTPA.

Task 6: In vitro testing of ^{111}In-HSA-hEGF bioconjugate for radiotoxicity against MDA-MB-468 and MCF-7 breast cancer cells.

The antiproliferative effects of ^{111}In-HSA-hEGF were evaluated *in vitro* against MDA-MB-468 breast cancer cells overexpressing EGFR (1-2 × 10^6 receptors/cell). The specific activity of ^{111}In-HSA-hEGF was increased 9-fold compared to ^{111}In-hEGF due to multiple substitution of HSA-hEGF with DTPA metal chelators for ^{111}In (7). The 9-fold higher specific activity yielded a 4-fold increased antiproliferative potency against MDA-MB-468 cells compared to ^{111}In-hEGF (IC_{50} 15 pM vs. 60 pM respectively).

SUMMARY OF RESEARCH ACCOMPLISHED IN YEAR 3 (2000-2001)

The following represents a summary of the research accomplished in the 3rd year of the project.

Task 4: Completion of studies examining the relative cytotoxicity of ^{111}In-hEGF, chemotherapy and external γ-radiation against EGFR-positive breast cancer cells.

Studies examining the cytotoxicity of ^{111}In-hEGF *in vitro* against breast cancer cells were completed by determining the antiproliferative effects of the radiopharmaceutical in combination with chemotherapeutic agents or external γ-radiation (8). MDA-MB-468 breast cancer cells (1-2 × 10^6 EGFR/cell) were seeded in triplicate into wells (10^3 cells/well) in a 96-well tissue culture plate. ^{111}In-hEGF was added to the wells (2-16 pM, 0.01-0.1 μCi/mL) and the cells were cultured for 5 days. Increasing concentrations of doxorubicin (25-100 nM), camptothecin (5-20 nM) or paclitaxel (Taxol®, 5-20 nM) in phosphate-buffered saline pH 7.4 (PBS) containing 0.1% DMSO diluted in growth medium were then added to the wells and the cells cultured for a further 2 days. Combination experiments with external γ-radiation were performed by seeding the MDA-MB-468 cells, culturing for 24 hours then treating the cells with 2-20 Gy of external γ-radiation using a ^{137}Cs source (1.1 Gy/minute). The cells were then cultured for a further 24 hours then treated with ^{111}In-hEGF for an additional 6 days. Control wells contained cells incubated with ^{111}In-hEGF alone. The proportion of cell growth inhibition was determined by the WST-1 colorimetric cell viability assay. The results of these experiments (**Fig. 1** and **Preprint 1**) demonstrated that the cytotoxic effects of chemotherapeutic agents or external γ-radiation were additive with those of ^{111}In-hEGF. It was shown that low concentrations of chemotherapeutic agents in combination with ^{111}In-hEGF increased the proportion of cell kill. The strongest effect was with doxorubicin and camptothecin. For example, 25 nM of doxorubicin combined with 4 pCi/cell of ^{111}In-hEGF resulted in more than a 2-fold improvement in cytotoxicity against MDA-MB-468 cells than for ^{111}In-hEGF alone. Similar results were obtained with external γ-radiation.

Combining 4 Gy of external γ-radiation with 5 pCi/cell of ^{111}In-hEGF improved the cytotoxicity 3-fold compaed to ^{111}In-hEGF alone.

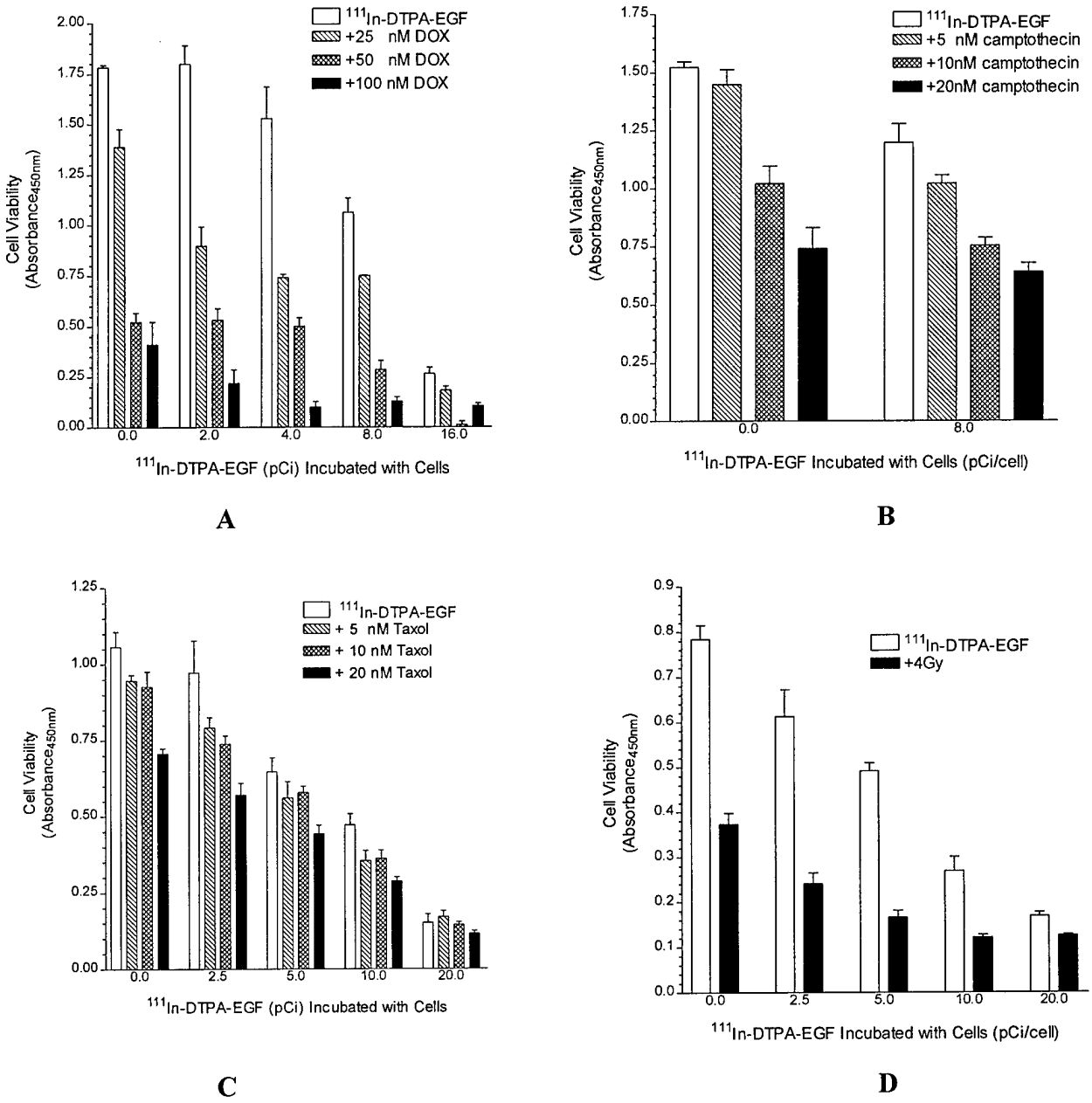


Fig. 1. Effect of treatment of MDA-MB-468 cells *in vitro* with ^{111}In-hEGF in combination with **A**. doxorubicin, **B**. camptothecin, **C**. paclitaxel or **D**. external γ-radiation. ^{111}In-DTPA-hEGF, "0.0" pCi/cell represents treatment with chemotherapeutic agents or γ-radiation alone. Values on the ordinate are absorbance values in the WST-1 colorimetric cell viability assay which are directly proportional to the number of viable cells.

Task 6: Completion of studies examining the in vitro radiotoxicity of ^{111}In-HSA-hEGF against MDA-MB-468 and MCF-7 breast cancer cells

To complete the studies examining the cytotoxicity of ^{111}In-HSA-hEGF against breast cancer cells, experiments were conducted to determine the selectivity of cytotoxicity of ^{111}In-HSA-hEGF *in vitro* against MDA-MB-468 human breast cancer cells overexpressing EGFR (1-2 × 10^6 receptors/cell) or MCF-7 breast cancer cells expressing a 100-fold lower level of EGFR on their surface (1 × 10^4 receptors/cell). MDA-MB-468 or MCF-7 cells were cultured *in vitro* with increasing concentrations (7.5-250 pM) of ^{111}In-HSA-hEGF or ^{111}In-hEGF. HSA-hEGF was derivatized with 9 DTPA metal chelators and labeled with ^{111}In to a specific activity of 42 MBq/μg (2.7 X 10^6 MBq/μmol). hEGF was derivatized with 1-2 DTPA metal chelators and labeled with ^{111}In to a specific activity 40 MBq/μg (2.4 × 10^5 MBq/μmol). Cell growth over a 7-day period was compared for treated cells and untreated cells using the WST-1 colorimetric cell viability assay. These experiments demonstrated that ^{111}In-HSA-hEGF was 4-fold more potent at inhibiting the growth of MDA-MB-468 cells than ^{111}In-hEGF exhibiting an IC_{50} of 15 pM vs. 60 pM respectively (**Fig. 2** and **Preprint 2**). ^{111}In-HSA-hEGF and ^{111}In-hEGF were also selectively cytotoxic to MDA-MB-468 breast cancer cells overexpressing EGFR but were not cytotoxic to MCF-7 breast cancer cells with a 100-fold lower level of EGFR expression (**Fig. 2**). ^{111}In-HSA-hEGF and ^{111}In-hEGF were slightly growth stimulatory to MCF-7 cells, particularly at concentrations >100 pM. This is expected since breast cancer cells with low levels of EGFR are growth-stimulated by EGF whereas cells with high levels of EGFR are growth-inhibited (6).

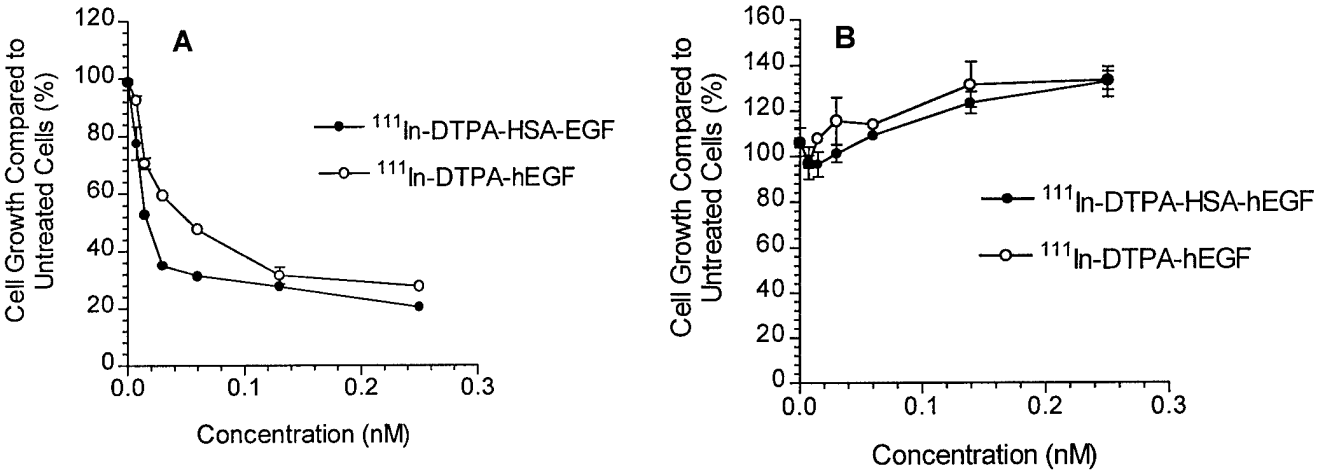


Fig. 2. Comparison of antiproliferative effects of ^{111}In-HSA-hEGF and ^{111}In-hEGF against **A.** MDA-MB-468 breast cancer cells or **B.** MCF-7 breast cancer cells.

Task 7: Treatment of mice implanted with subcutaneous EGFR-positive MDA-MB-468 xenografts with ^{111}In-HSA-hEGF or ^{111}In-hEGF

The anti-tumor effects and normal tissue toxicity of ^{111}In-hEGF were evaluated *in vivo* in athymic mice implanted with subcutaneous MDA-MB-468 human breast cancer xenografts (**Fig. 3** and **Reprint 3**). Groups of 5 mice received five weekly s.c. injections of 500 μCi (17 μg) of

^{111}In-hEGF (total 2.5 mCi). Control animals received injections of unlabeled hEGF (17 μg) or normal saline. ^{111}In-hEGF completely arrested the growth of MDA-MB-468 tumors (**Fig. 3-A**). Interestingly, treatment of mice with doxorubicin (5 mg/kg × 2 days) produced only a 2-fold tumor growth inhibition (not shown). Although tumors were only arrested by ^{111}In-hEGF in this study, more recent experiments have shown that the anti-tumor effects of the radiopharmaceutical are tumor-size dependent and tumor regression can be achieved if ^{111}In-hEGF treatment is started early when tumors are small (4 mm^3) rather than large (21 mm^3) (9). This effect is probably due to improved uptake and penetration of the radiopharmaceutical in small tumors. Normal tissue toxicity was evaluated by monitoring whole body weight, serum alanine aminotransferase (ALT) and creatinine (SCr) levels, peripheral blood counts and hemoglobin and by histopathological examination of normal tissues by light and electron microscopy (EM). There was no significant change in body weight in mice treated with ^{111}In-hEGF (**Fig. 3-B**). Body weight was identical to that for control mice treated with unlabeled hEGF or normal saline, suggesting that there was no generalized normal tissue toxicity associated with the radiopharmaceutical. EM studies performed by Dr. Ross Cameron (a board-certified pathologist and co-investigator) of the liver and kidneys (the only normal tissues to express moderate levels of EGFR, ~10^5 receptors/cell) revealed no evidence of morphological damage (**Fig. 4**). In contrast, mice treated with doxorubicin had a 20% decrease in body weight and 2/5 mice died of treatment-related toxicity. Extensive cardiotoxicity was also noted in mice treated with doxorubicin. No mice died of toxicity in the ^{111}In-hEGF group.

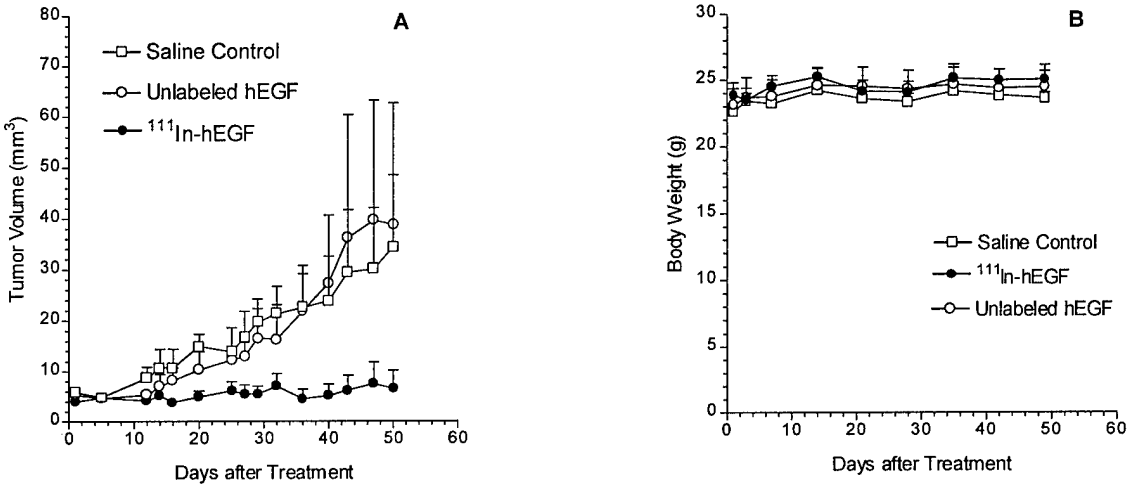


Fig. 3. A. Tumor growth in athymic mice implanted with s.c. MDA-MB-468 breast cancer xenografts treated with five weekly s.c. injections of 500 μCi (17 μg) of ^{111}In-hEGF, unlabeled hEGF (17 μg) or normal saline. ^{111}In-hEGF completely arrested tumor growth in comparison to control animals. **B.** There was no change in body weight in mice treated with ^{111}In-hEGF or in control mice treated with normal saline or unlabeled hEGF.

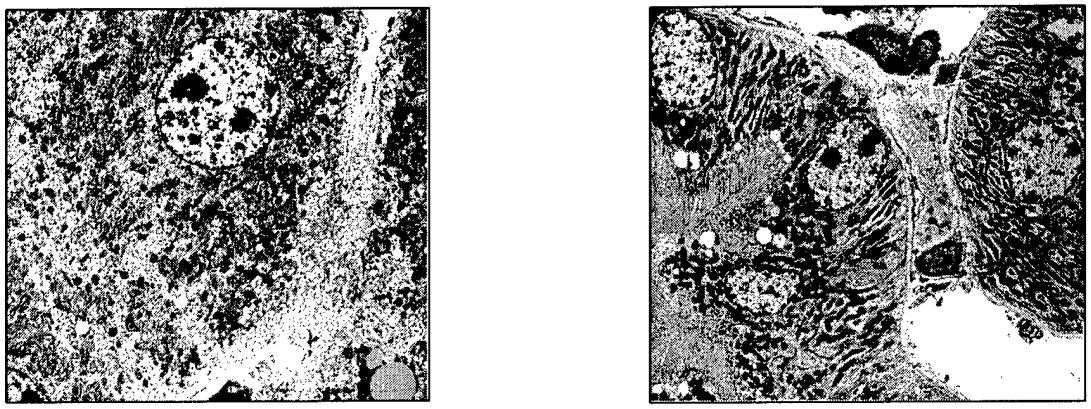
There was also no increase in ALT or SCr in mice treated with ^{111}In-hEGF compared to control mice treated with saline (**Table 1**) confirming the absence of hepatotoxicity and renal toxicity.

Table 1. Clinical biochemistry and hematology values in mice treated with ^{111}In-hEGF*.

	Normal Saline Control Group	^{111}In-hEGF Treatment Group
ALT	16-40 units/L	18-46 units/L
SCr	60-66 μmol/L	60-78 μmol/L
WBC	12.4×10^9/L	5.8×10^9/L †
Platelets	120.8×10^9/L	88.5×10^9/L ‡
RBC	8.9×10^{12}/L	8.2×10^{12}/L
Hemoglobin	143 g/L	136 g/L

**Values shown are means or range for a group of 4-5 animals. † Within normal range (4.5-10 × 10^9/L). ‡ Lower than controls but not clinically significant.*

There was a modest decrease in WBC and platelets in mice treated with ^{111}In-hEGF, but WBC values remained within the normal range and the decrease in platelet counts would not be clinically significant in humans. There was no decrease in RBC or hemoglobin with ^{111}In-hEGF. The reason for decreased WBC and platelet counts is not known at present but may be due to non-specific irradiation of the bone marrow by the penetrating γ-emissions of ^{111}In rather than specific radiotoxicity mediated by the short-range Auger electron emissions, since <3% of bone marrow stem cells are EGFR-positive (3) and capable of binding and internalizing ^{111}In-hEGF.



A **B**

Fig. 4. Electron microscopy of **A**. liver or **B**. kidneys in mice treated with 5 weekly s.c. injections of 500 μCi of ^{111}In-hEGF. There was no evidence of morphological damage to the liver, ie. mitochondrial changes, proliferation of smooth endoplasmic reticulum or lysosomes or steatosis. Similarly, there was no damage to renal tubular structures in the kidneys.

PLANNED RESEARCH FOR YEAR 4 (2001-2002)

Task 7: Treatment of mice implanted with subcutaneous EGFR-positive MDA-MB-468 xenografts with ^{111}In-hEGF

We plan to continue our experiments examining the anti-tumour effects and normal tissue toxicity of ^{111}In-hEGF in mice implanted with subcutaneous human breast cancer xenografts. In particular, we are interested in determining the selectivity of the treatment for EGFR-

overexpressing breast cancer by comparing the antitumour effects of the radiopharmaceutical in mice implanted with MDA-MB-468 breast cancer xenografts (1-2 × 10^6 EGFR/cell) or MCF-7 breast cancer xenografts (1 × 10^4 EGFR/cell). We also intend to examine the toxicity of the radiopharmaceutical against peripheral blood leukocytes and platelets more closely, since we observed a slight decrease in these values at the end of a 6-8 week treatment period. We plan on measuring the effect of ^{111}In-hEGF at earlier and more frequent intervals in order to determine the kinetics of toxicity against leukocytes and platelets. Our initial experiments further suggest that the effectiveness of the radiopharmaceutical is tumour-size dependent with smaller tumours exhibiting a greater response than larger tumours. We wish to evaluate this phenomenon in more detail by evaluating the antitumour effects of ^{111}In-hEGF in animals implanted with different sized breast cancer xenografts.

Task 8: Treatment of mice implanted with subcutaneous EGFR-positive MDA-MB-468 xenografts with ^{111}In-hEGF in comparison with chemotherapy.

In the final year of the project, we are also planning to compare the antitumour effects and normal tissue toxicity of ^{111}In-hEGF with that produced by doxorubicin, one of the most commonly used chemotherapeutic agents for advanced breast cancer. Mice implanted with MDA-MB-468 human breast cancer xenografts will be treated with ^{111}In-hEGF (6 doses of 500 μCi) as previously described or a standard regimen of doxorubicin (5 mg/kg × 2 days administered intraperitoneally). Tumour volume will be monitored over an 8-week period and compared to that observed for untreated mice to obtain a tumour growth index. Comparisons of tumour growth indices will then be made between ^{111}In-hEGF treatment and the doxorubicin groups. Normal tissue toxicity will be assessed by monitoring whole body weight, serum ALT and creatinine measurements, histopathological examination of tissues and peripheral blood counts as previously described.

Task 9: Preparation and submission of manuscripts reporting the results of the research.

In the final year of the research project, we will also prepare and submit several manuscripts documenting our research to peer-reviewed journals. We have already submitted one manuscript in May 2001 to *Nuclear Medicine and Biology* on the construction and *in vitro* testing of the ^{111}In-HSA-hEGF bioconjugate (**Preprint 1**). A revised version of the manuscript was recently re-submitted to the journal and we expect that it will be approved for publication shortly. We intend to submit a second manuscript on a comparison of the antiproliferative effects *in vitro* of ^{111}In-hEGF, chemotherapy and external γ-radiation on EGFR-positive breast cancer cells to the *International Journal of Cancer* by September 2001 (**Preprint 2**). A third manuscript describing the antitumour effects and normal tissue toxicity in mice implanted with human breast cancer xenografts is in preparation and we expect to submit this manuscript by January 2002.

KEY RESEARCH ACCOMPLISHMENTS

- Completed *in vitro* testing of ^{111}In-hEGF in comparison and in combination with chemotherapeutic agents or external γ-radiation against human breast cancer cells overexpressing EGFR. These studies clearly demonstrated the greater potency of ^{111}In-hEGF compared to chemotherapeutic agents against breast cancer cells but also showed that

increased cytotoxicity could be achieved by combining ^{111}In-hEGF with low dose chemotherapy or external γ-radiation.

- Completed *in vitro* testing of ^{111}In-HSA-hEGF against MDA-MB-468 breast cancer cells overexpressing EGFR (1-2 × 10^6 receptors/cell) or MCF-7 breast cancer cells expressing low levels of EGFR (1 × 10^4 receptors/cell). These studies demonstrated that ^{111}In-HSA-hEGF exhibited selective cytotoxicity against breast cancer cells overexpressing EGFR but was slightly growth stimulatory for breast cancer cells expressing low levels of EGFR.

- Conducted first *in vivo* testing of ^{111}In-hEGF for treatment of MDA-MB-468 breast cancer xenografts implanted subcutaneously in athymic mice. These studies demonstrated that ^{111}In-hEGF completely arrested the growth of breast cancer tumours in the animals, whereas treatment with unlabeled hEGF or normal saline had no effect on tumour growth. There was no evidence of normal tissue toxicity particularly to the liver or kidneys which express moderate levels of EGFR, but there was a slight decrease in peripheral blood leukocytes and platelets. The antitumour effects of ^{111}In-hEGF were tumour-size dependent with smaller tumours responding more effectively than larger tumours.

REPORTABLE OUTCOMES

Manuscripts

1. Wang J, Chen P, Su ZF, Vallis K, Sandhu J, Hendler A, Cameron R and Reilly RM. Amplified delivery of indium-111 to EGFR-positive breast cancer cells. *Nucl. Med. Biol.* (submitted), 2001. [Senior responsible author].

2. Chen P, Mrkobrada M, Vallis K, Sandhu J, Cameron R, Hendler A and Reilly RM. Comparative antiproliferative effects of novel targeted Auger electron radiotherapy, γ-radiation or chemotherapy on breast cancer cells overexpressing EGFR. *Int. J. Cancer* (submitted), 2001. [Senior responsible author].

3. Reilly RM, Kiarash R, Sandhu J, Lee YW, Cameron R, Hendler A, Vallis K and Gariépy J. A comparison of epidermal growth factor and monoclonal antibody 528 labeled with indium-111 for imaging human breast cancer. *J. Nucl. Med.* 41: 903-911, 2000. [Principal author].

4. Reilly RM, Kiarash R, Cameron R, Porlier N, Sandhu, J, Vallis K, Hendler A, Hill RP and Gariépy J. Indium-111 EGF is selectively radiotoxic to human breast cancer cells overexpressing EGFR. *J. Nucl. Med.* 41: 429-438, 2000. [Principal author].

Abstracts

1. Chen, P., Cameron, J., Wang, J., Vallis, K., Sandhu, J., Hendler, A.L. and Reilly, R..M. The anti-tumor effect of the Auger electron-emitting radiopharmaceutical, ^{111}In-hEGF against MDA-MB-468 human breast cancer xenografts is tumor-size dependent. *J. Nucl. Med.* 45: 255P [Abstract 1073]. [Senior responsible author].

2. Chen, P., Brandwein, J. Wedel, N., O'Connor, J. and Reilly, R.M. ^{111}In-labeled humanized monoclonal antibody HuM195 is selectively radiotoxic to human leukemia cells expressing CD33. *J. Nucl. Med.* 45: 255P [Abstract 1072]. [Senior responsible author].

3. Chen, P., Mrkobrada, M., Vallis, K. and Reilly, R.M. Comparison of the cytotoxic effects of targeted Auger electron radiotherapy, external γ-radiation and cancer chemotherapeutics against EGFR-positive human breast cancer cells. *Proc. American Assoc. Cancer Research* 91st Annual Meeting 2000. [abstract] [Senior responsible author].

4. Wang J., Sandhu, J., Chen Z., Leung C., Bray M., Yang M., Cameron R., Hendler A., Vallis K., Reilly R.M. Production of a human epidermal growth factor (hEGF)-immunoglobulin (C_H1) fusion protein for targeting human breast cancer. *Clin. Cancer Res.* 1999 [abstract] [Senior responsible author].

5. Wang J., Sandhu, J., Chen Z., Leung C., Bray M., Yang M., Cameron R., Hendler A., Vallis K., Reilly R.M. Production of a human epidermal growth factor (hEGF)-immunoglobulin (C_H1) fusion protein for targeting human breast cancer. *J. Nucl. Med.* 1999 [abstract] [Senior responsible author].

Presentations

1. Reilly, R.M. Molecular imaging and targeted radiotherapy of cancer: Past, present and future. Presented at Department of Medical Biophysics graduate student seminar, Sunnybrook and Women's College Health Sciences Centre, Toronto, ON, July 4, 2001.

2. Reilly, RM. Targeted Auger electron radiotherapy of malignancies. Presented at the Ontario Association of Medical and Radiological Technologists meeting, Richmond Hill, May 5, 2001. [Principal author].

3. Reilly, RM. Targeted Auger electron radiotherapy of malignancies. Presented at Nuclear Medicine Rounds, University Hospital, London, ON, April 19, 2000 [Principal author]

4. Reilly RM. The Trojan Horse: Targeted auger electron radiotherapy of breast cancer. Presented at Department of Radiation Oncology Rounds, University of Toronto, February 25, 1999 [Principal author]

Media Coverage

1. National Post Newspaper, Toronto, June 25, 2001. *Scientists Test New Cancer Weapon.* Heather Sokoloff. Story about preclinical testing of a new radiopharmaceutical treatment for advanced breast cancer.

Applications for Funding Based on Research

U.S. Army Breast Cancer Research Program. 2002-2004. R.M. Reilly (P.I.), K. A. Vallis, A. Oza, G. Lockwood, A. Hendler, R. Cameron. Preclinical lead-up studies in support of an IND application for ^{111}In-hEGF, A new radiopharmaceutical for treatment of advanced breast cancer. $ 199,595 U.S. applied.

Susan G. Komen Breast Cancer Foundation. 2001-2003. K. A. Vallis (P.I.), R.M. Reilly (Co.I.), A. Oza, G. Lockwood, A. Hendler, R. Cameron, W. Wells and D. Warr. A Phase I Study of ^{111}In-Epidermal Growth Factor. A Novel Radiopharmaceutical Agent for the Treatment of Breast Cancer. $ 250,000 U.S. applied.

James Birrell Neuroblastoma Research Fund. 2001-2002. R.M. Reilly (P.I.) and S. Baruchel (Co.I.). Novel targeted Auger electron radiotherapy of neuroblastoma using ^{123}I-MIBG. $ 25,000 awarded.

Natural Sciences and Engineering Research Council of Canada 2000-2003. Vascular growth factor receptors as a target for Auger electron radiotherapy of malignant astrocytomas. J. Sandhu, R.M. Reilly (Co.I.) and A. Guha. $ 252,000 awarded.

CONCLUSIONS AND FUTURE RESEARCH

We conclude that ^{111}In-hEGF is a highly promising new radiotherapeutic agent for advanced, estrogen receptor-negative, hormone-resistant breast cancer. The radiopharmaceutical is highly and selectively cytotoxic *in vitro* to human breast cancer cells overexpressing EGFR. ^{111}In-hEGF exhibits strong antiproliferative effects on breast cancer cells *in vitro* at concentrations at least 100-500 fold lower than those required for commonly used chemotherapeutic drugs. Very low concentrations of ^{111}In-hEGF (<70 pM) produced the same growth inhibitory effects as 4 Gy of high dose rate external γ-radiation. The cytotoxicity of the radiopharmaceutical is amplified by conjugation of hEGF with HSA and derivatization of the resulting bioconjugate with multiple DTPA metal chelators for ^{111}In. Treatment of mice implanted subcutaneously with human breast cancer xenografts overexpressing EGFR with 5 × 500 μCi doses of ^{111}In-hEGF completely arrested the growth of the tumours in comparison to control mice treated with unlabeled hEGF or normal saline. There was no significant change in body weight following treatment with ^{111}In-hEGF indicating no generalized normal tissue toxicity. There was also no evidence of damage to the liver or kidneys by electron microscopy and by measuring serum ALT and creatinine. There was a slight, but not clinically significant decrease in peripheral blood leukocytes and platelets. Based on these highly promising preclinical results with ^{111}In-hEGF against breast cancer cell lines and tumour xenografts supported by the *IDEA Award*, we are now planning a Phase I clinical trial of the radiopharmaceutical in breast cancer patients, which we hope to commence at the University Health Network by the end of 2002. An application has been submitted to the U.S. Army Breast Cancer Research Program for a *Clinical Bridge Award* to support the development of a clinical quality formulation and to collect supporting data for an Investigational New Drug (IND) application to Health Canada for ^{111}In-hEGF. Support for the Phase I clinical trial may be requested either from the Susan G. Komen Breast Cancer Foundation or submitted to the U.S. Army Breast Cancer Research Program as a *Clinical Translational Research Award* application.

REFERENCES

1. Klijn JGM, Berns PMJ, Schmitz PIM, Foekens JA. The clinical significance of epidermal growth factor receptor (EGF-R) in human breast cancer: a review on 5232 patients. *Endocr. Rev.* 1992; 13: 3-17.

2. Rakowicz-Szulcynska EM, Rodeck U, Herlyn M, Koprowski H. Chromatin binding of epidermal growth factor, nerve growth factor, and platelet-derived growth factor in cells bearing the appropriate surface receptors. *Proc. Natl. Acad. Sci.* USA 1986; 83: 3728-3732.

3. Waltz TM, Malm C, Nishikawa BK, Wasteson A. Transforming growth factor-alpha (TGF-alpha) in human bone marrow: demonstration of TGF-alpha in erythroblasts and eosinophilic precursor cells and of epidermal growth factor receptors in blastlike cells of myelomonocytic origin. *Blood* 1995; 85: 2385-2392.

4. Dunn WA, Hubbard AL. Receptor-mediated endocytosis of epidermal growth factor by hepatocytes in the perfused rat liver: ligand and receptor dynamics. *J. Cell Biol.* 1984; 98: 2148-2159.

5. Fisher DA, Salido EC, Barajas L. Epidermal growth factor and the kidney. *Ann. Rev. Physiol.* 1989; 51: 67-80.

6. Filmus J, Pollak MN, Cailleau R, Buick RN. A human breast cancer cell line with a high number of epidermal growth factor (EGF) receptors, has an amplified EGF receptor gene and is growth inhibited by EGF. *Biochem Biophys Res Commun* 1985; 128: 898-905.

7. Wang J, Chen P, Su Z-F, Vallis K, Sandhu J, Cameron R, Hendler A and Reilly RM. Amplified delivery of indium-111 to EGFR-positive breast cancer cells. *Nucl. Med. Biol.* 2001 (submitted).

8. Chen P, Mrkobrada M, Vallis KA, Sandhu J, Cameron R, Hendler A and Reilly RM. Comparative antiproliferative effects of novel targted Auger electron radiotherapy, chemotherapy and external γ-radiation on EGFR-overexpressing breast cancer cells. *Int J. Cancer* 2001 (submitted).

9. Chen, P., Cameron, J., Wang, J., Vallis, K., Sandhu, J., Hendler, A.L. and Reilly, R..M. The anti-tumor effect of the Auger electron-emitting radiopharmaceutical, ^{111}In-hEGF against MDA-MB-468 human breast cancer xenografts is tumor-size dependent. *J. Nucl. Med.* 45: 255P [Abstract 1073].

APPENDICES

Comparative Antiproliferative Effects of Novel Targeted Auger Electron Radiotherapy, Chemotherapy and External γ-Radiation on EGFR-Overexpressing Breast Cancer Cells[1]

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Running Title: Targeted Auger Electron Radiotherapy of Breast Cancer

Key Words: breast cancer; Auger electrons; chemotherapy; radiotherapy; epidermal growth factor receptor

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[3] The abbreviations used are: FCS, fetal calf serum; DMSO, dimethylsulfoxide; hEGF, human epidermal growth factor; EGFR, epidermal growth factor receptor; PBS, phosphate buffered saline; 5-FU, 5-fluorouracil; MTX, methotrexate; mAb, monoclonal antibody; RIT, radioimmunotherapy

ABSTRACT

Targeted radiotherapy using radiopharmaceuticals labeled with low energy, short-range Auger electron emitters is a promising novel treatment strategy for cancer. We previously demonstrated that human EGF labeled with the Auger electron emitter, ^{111}In (^{111}In-DTPA-hEGF) was highly and selectively radiotoxic to MDA-MB-468 human breast cancer cells overexpressing EGFR. Our objective in this study was to directly compare the antiproliferative effects *in vitro* of ^{111}In-DTPA-hEGF against MDA-MB-468 cells with those produced by chemotherapeutic agents or external γ-radiation. MDA-MB-468 cells were treated *in vitro* with ^{111}In-DTPA-hEGF (12.5-200 pM, 0.02-1 μCi/mL), unlabeled hEGF (12.5-200 pM), paclitaxel (0.8-25 nM), methotrexate (15-120 nM), doxorubicin (3-200 nM), camptothecin (3-100 nM), 5-FU (1-20 μM) or γ-radiation (2-20 Gy). Cell growth was determined colorimetrically (WST-1 assay) after a culture period of 7 days. Treatments combining ^{111}In-DTPA-hEGF with doxorubicin, paclitaxel or γ-radiation were also studied. Apoptosis or necrosis of MDA-MB-468 cells treated with ^{111}In-DTPA-hEGF or unlabeled hEGF was evaluated by flow cytometry using fluorescein-Annexin V or propidium iodide staining respectively. Cell cycle kinetics and DNA fragmentation (Comet assay) were studied in cells treated with ^{111}In-DTPA-hEGF or γ-radiation. Our results revealed that ^{111}In-DTPA-hEGF was several logarithms more potent on a molar concentration basis than chemotherapeutic agents at inhibiting the growth of MDA-MB-468 cells. The IC_{50} value for ^{111}In-DTPA-hEGF was <70 pM (0.3 μCi/mL), whereas IC_{50} values for chemotherapeutic agents ranged from 6 nM (paclitaxel) to 4 μM (5-FU). The IC_{50} for unlabeled hEGF was >200 pM. The antiproliferative effect of 70 pM (0.3 μCi/mL) of ^{111}In-DTPA-hEGF was equivalent to that produced by 4 Gy of external γ-radiation. Treatment of MDA-MB-468 cells with ^{111}In-DTPA-hEGF combined with doxorubicin or paclitaxel increased the antiproliferative effect 2-5 fold. Treatment of MDA-MB-468 cells with ^{111}In-DTPA-

hEGF combined with γ-radiation enhanced cell growth inhibition 8-fold. The proportion of apoptotic cells was increased 250-fold following exposure to ^{111}In-DTPA-hEGF and the fraction of necrotic cells was increased 60-fold. Treatment of MDA-MB-468 cells with ^{111}In-DTPA-hEGF caused G2/M-phase arrest. Extensive DNA fragmentation was observed. We conclude that Auger electron-emitting radiopharmaceuticals are more effective on a molar concentration basis than chemotherapeutic agents at inhibiting the growth of EGFR-overexpressing breast cancer cells *in vitro* and are highly promising radiotherapeutic agents for the treatment of advanced breast cancer in humans.

INTRODUCTION

Early detection of breast cancer has improved the prognosis for patients with primary disease confined to the breast but the prognosis for patients with advanced, metastatic breast cancer remains poor. New anticancer treatments which exploit novel mechanisms of cytotoxicity are needed to make an impact on the survival and quality of life of these patients. Targeted radiotherapy using Auger electron-emitting radiopharmaceuticals is one promising novel therapeutic strategy currently being explored for treatment of malignancies (1,2). Auger electrons are highly damaging to chromosomal DNA when the radiopharmaceuticals are internalized into the cytoplasm and particularly when imported into the nucleus of cancer cells. Furthermore, the subcellular range of the electrons should in theory minimize or potentially eliminate non-specific normal tissue radiotoxicity (ie. against bone-marrow stem cells) previously found to be dose-limiting for radiopharmaceuticals labeled with longer range β-emitters (eg. ^{131}I or ^{90}Y labeled mAb) (3).

EGF is a 6 kDa specific peptide ligand for the EGFR, a 170 kDa transmembrane receptor tyrosine kinase overexpressed on 30-50% of human breast cancers [reviewed in (4)]. EGFR overexpression in breast cancer is associated with a poor long-term survival, shortened time to recurrence and an inadequate response to hormonal therapy (4). We previously reported that human EGF (hEGF) conjugated to the Auger electron-emitting radionuclide, ^{111}In was rapidly bound, internalized and translocated to the cell nucleus in EGFR-positive breast cancer cells (5). The radiopharmaceutical was highly radiotoxic to MDA-MB-468 breast cancer cells overexpressing EGFR (1-2 × 10^6 receptors/cell) but was not radiotoxic to MCF-7 breast cancer cells exhibiting a 100-fold lower level of EGFR expression. Importantly, ^{111}In-hEGF was not radiotoxic to normal tissues such as the liver and kidneys which express moderate levels of EGFR, when high amounts

of the radiopharmaceutical (equivalent to human doses up to 400 mCi) were administered to mice (5).

In this study, we describe for the first time a direct comparison of the antiproliferative effects *in vitro* of targeted Auger electron radiotherapy using ^{111}In-DTPA-hEGF against MDA-MB-468 cells with those produced by selected chemotherapeutic agents or external γ-radiation. Our results reveal that ^{111}In-DTPA-hEGF is at least 100-400 fold more potent on a molar concentration basis than paclitaxel, doxorubicin, camptothecin or MTX and up to 4-logarithms more potent than 5-FU at inhibiting the growth of MDA-MB-468 cells. Picomolar concentrations of the radiopharmaceutical produced an antiproliferative effect equivalent to that of several Gy of external γ-radiation. Treatment of MDA-MB-468 cells with ^{111}In-DTPA-hEGF combined with chemotherapy or external γ-radiation was more effective at inhibiting the growth of MDA-MB-468 cells than either treatment alone. These findings are encouraging for further investigation of the antitumor effects of targeted Auger electron radiotherapy against EGFR-overexpressing breast cancer *in vivo*, alone or in combination with conventional cancer treatment modalities.

MATERIALS AND METHODS

Breast cancer cells. MDA-MB-468 human breast cancer cells were obtained from the American Type Culture Collection (Manassas, VA) and were cultured in L-15 medium (Sigma, St. Louis, MO) containing 100 units/mL of penicillin, 100 μg/mL of streptomycin, 2 mM L-glutamine and supplemented with 10% FCS. MDA-MB-468 cells express 1-2 × 10^6 EGFR/cell (6).

Labeling of hEGF with ^{111}In. hEGF (Upstate Biotechnology, Lake Placid, NY) was derivatized with DTPA and labeled to high specific activity (800 μCi/μg, 4.8×10^6 μCi/μmol) with ^{111}In acetate as previously described (5). The radiochemical purity of ^{111}In-DTPA-hEGF was 95-98% measured by ITLC-SG in 100 mM sodium citrate pH 5.0. ^{111}In-DTPA-hEGF exhibited preserved receptor-binding properties against MDA-MB-468 cells in a direct radioligand binding assay (K_a 7.5×10^8 L/mol; B_{max} 1.3×10^6 EGFR/cell) (7). ^{111}In-DTPA-hEGF was sterilized by filtration through a Millex GV 0.22 μm filter (Millipore, Bedford, MA).

Chemotherapeutic Agents. Paclitaxel, doxorubicin, 5-FU, MTX and camptothecin were obtained from Sigma and were dissolved at an initial concentration of 10 mM in PBS pH 7.4 containing 0.1% DMSO (cell culture grade) and then stored at 4 °C. Immediately prior to use, the solutions were diluted with serum-free medium to the final concentration to be evaluated and sterilized by filtration through a Millex GV 0.22 μm filter.

Treatment of Breast Cancer Cells with ^{111}In-DTPA-hEGF. Subconfluent MDA-MB-468 cells in exponential growth phase were seeded in triplicate into wells (10^3 cells/well) in a 96-well tissue culture plate (Nunclon, Canadian Life Technologies, Burlington, ON). Growth medium was added to each well (100 μL/well) and the cells were cultured for 24 h. The cells were incubated with ^{111}In-DTPA-hEGF (4-200 pM, 0.02-1 μCi/mL), unlabeled hEGF (4-200 pM) or PBS pH 7.4 (negative control) diluted with growth medium and cultured for an additional 7 days. The proportion of cell growth inhibition was determined by the WST-1 colorimetric cell viability assay (Boehringer-Mannheim, Laval, PQ).

Treatment of Breast Cancer Cells with Chemotherapeutic Agents. MDA-MB-468 cells were seeded in triplicate into wells (10^3 cells/well) in a 96-well tissue culture plate and treated with paclitaxel (1-25 nM), doxorubicin (3-200 nM). 5-FU (1-20 μM), MTX (15-120 nM) or camptothecin (3-100 nM) in PBS pH 7.4 containing 0.1% DMSO diluted with growth medium. Negative control wells contained cells incubated with PBS pH 7.4 containing 0.1% DMSO diluted with growth medium. The growth inhibitory properties of ^{111}In-DTPA-hEGF (17-21 pM, 0.1 μCi/mL) in combination with paclitaxel (20 nM) or doxorubicin (100 nM) were also studied by adding the chemotherapeutic agent directly to the wells after 5 days of incubation with ^{111}In-DTPA-hEGF and culturing the cells for an additional 2 days. Control wells for combination experiments contained cells treated with ^{111}In-DTPA-hEGF alone. The proportion of cell growth inhibition was determined by the WST-1 colorimetric cell viability assay.

Treatment of Breast Cancer Cells with External γ-Radiation. MDA-MB-468 cells were seeded in triplicate into wells (10^3 cells/well) in a 96-well tissue culture plate and cultured for 24 h. The medium was replaced with fresh medium and adherent cells were treated with external γ-radiation (2-20 Gy) delivered at 1.1 Gy/min using a ^{137}Cs source (GC-40E cell irradiator, MDS-Nordion Inc., Kanata, ON). The cells were then cultured for a period of 7 days. Negative control wells contained non-irradiated cells cultured in growth medium. The growth inhibition of MDA-MB-468 cells treated with a combination of external γ-radiation and ^{111}In-DTPA-hEGF was investigated by seeding cells into 96-well plates (10^3 cells/well), culturing for 24 h, then treating the cells with 2-8 Gy of γ-radiation. The cells were cultured for a further 24 h, then incubated with ^{111}In-DTPA-hEGF (100 pM, 0.5 μCi/mL) in growth medium for 6 days.

WST-1 Colorimetric Cell Viability Assay. Growth inhibition of MDA-MB-468 cells treated with ^{111}In-DTPA-hEGF, chemotherapeutic agents or γ-radiation was evaluated using a colorimetric cell viability assay (WST-1, Boehringer-Mannheim, Laval, PQ). The cells were treated as described, then the number of viable cells at the end of the culture period was quantitated by adding WST-1 reagent (10 μL) directly into the wells and incubating the plates for 2 h at 37 °C. The absorbance of the colored formazan complex formed was measured at 450 nm in a plate reader (Bio-Tek Model ELx800, Winooski, VT). The absorbance of wells containing treated cells was compared to that for control wells containing untreated cells to determine the proportion of cell growth inhibition. The concentration of ^{111}In-DTPA-hEGF or chemotherapeutic agents required to cause 50% (IC_{50}) or 90% (IC_{90}) growth inhibition was estimated. Similarly the amount of γ-radiation (Gy) resulting in a 2-fold (ED_{50}) or 10-fold (ED_{90}) decrease in the growth of the cells was determined.

Evaluation of Apoptosis and Necrosis. Apoptosis in MDA-MB-468 cells treated with ^{111}In-DTPA-hEGF or unlabeled hEGF was studied by flow cytometry using fluorescein-Annexin V (8) (Annexin V-FITC Apoptosis Detection Kit, Sigma Chemical Co., St. Louis, MO). Necrosis was measured by intracellular accumulation of propidium iodide. MDA-MB-468 cells were cultured in growth medium for 4 days containing ^{111}In-DTPA-hEGF (200 pM, 1 μCi/mL), unlabeled hEGF (200 pM) or growth medium alone. The cells were recovered using trypsin/EDTA, washed with PBS pH 7.4 and resuspended in binding buffer (10 mM Hepes/NaOH pH 7.5 containing 140 mM NaCl and 2.5 mM $CaCl_2$). Fluorescein-Annexin V reagent (5 μL) and propidium iodide reagent (10 μL) were added to 500 μL of cell suspension (5×10^5 cells) and the cells were incubated in the dark for 10 min at room temperature. MDA-MB-468 cells were sorted by flow cytometry using a Epics XL-MCL flow cytometer (Beckman-Coulter, Palo Alto, CA).

Evaluation of Cell Cycle Kinetics. The effect of treatment of MDA-MB-468 cells with ^{111}In-DTPA-hEGF (250 pM, 1 μCi/mL), unlabeled hEGF (250 pM), or external γ-radiation (10 Gy) on cell cycle kinetics was determined by flow cytometry. Treated cells were harvested using trypsin/EDTA, centrifuged at 300 × *g* for 5 min and fixed in ice-cold 70% ethanol for 30 min. Fixed cells were centrifuged again at 300 × *g* for 5 min, rinsed two times with PBS pH 7.4 and incubated at 37 °C for 30 min in the dark with containing 500 μg/mL ribonuclease A (Sigma) and 5 μg/mL propidium iodide (Sigma) in PBS pH 7.4. The cells were washed 3 times with PBS pH 7.4 and sorted by flow cytometry. The proportion of cells in G1, S, or G2/M-phases was calculated using ModFit LT software (9).

Evaluation of DNA Fragmentation. DNA fragmentation was qualitatively evaluated in MDA-MB-468 cells treated with ^{111}In-DTPA-hEGF, unlabeled hEGF or external γ-radiation using a Comet assay (10). Briefly, cells were cultured for 4 days in growth medium containing ^{111}In-DTPA-hEGF (80 pM, 0.4 μCi/mL), unlabeled hEGF (80 pM) or external γ-radiation (10 Gy). The cells were recovered using trypsin/EDTA and suspended in 1% LMP agarose at 55 °C. A 300 μL aliquot of warm cell suspension was dispensed onto a frosted glass slide precoated with 1% LMP agarose. The slides were placed into cell lysing solution (1% sodium dodecylsulfonate containing 25 mM disodium EDTA pH 8.5) for 2 h in the dark at room temperature, then rinsed with TBE buffer (90 mM Tris base, 90 mM boric acid and 2 mM EDTA) pH 8.3 for 3 h in the dark at room temperature. The slides were electrophoresed in TBE buffer at a constant voltage of 22 volts. Electrophoresed slides were rinsed two times in distilled water and stained by incubation in propidium iodide (1

μg/mL in PBS pH 7.4, Sigma Chemical Co.) at room temperature for 30 min. After destaining in distilled water for 30 min, the slides were examined on a fluorescence microscope.

Results

Treatment of Breast Cancer Cells with ^{111}In-DTPA-hEGF. The growth of MDA-MB-468 cells was inhibited 10-fold at a concentration of <200 pM (1 μCi/mL) of ^{111}In-DTPA-hEGF (Table 1). Unlabeled hEGF was also slightly growth inhibitory for MDA-MB-468 cells causing a 1.5-fold decrease in the growth of the cells at 200 pM (results not shown). The IC_{50} for ^{111}In-DTPA-hEGF was 70 pM (0.3 μCi/mL, Table 1 and Fig. 1) and for unlabeled hEGF was >200 pM. The IC_{90} was not determined for unlabeled hEGF. These results confirmed our previous findings using a clonogenic assay that ^{111}In-DTPA-hEGF is highly radiotoxic to MDA-MB-468 breast cancer cells overexpressing EGFR (5).

Treatment of Breast Cancer Cells with Chemotherapeutic Agents. Paclitaxel was the most potent chemotherapeutic agent exhibiting an IC_{50} of 6 nM and an IC_{90} of 20 nM (Table 1). MTX was also highly effective at inhibiting the growth of MDA-MB-468 cells with an IC_{50} of 15 nM and an IC_{90} of 70 nM. The antiproliferative effects of doxorubicin and camptothecin were similar with IC_{50} values of 20-30 nM and IC_{90} values of 75 nM, but these agents were 10-15 fold less effective than paclitaxel in inhibiting the growth of MDA-MB-468 cells. 5-FU was the least potent chemotherapeutic agent with an IC_{50} of 4 μM and IC_{90} >10 μM (Table 1). Importantly, on a molar concentration basis, chemotherapeutic agents were more than 2-logarithms less potent than ^{111}In-DTPA-hEGF in inhibiting the growth of MDA-MB-468 cells (Fig. 1). A 300-400 fold higher concentration of doxorubicin or camptothecin, or a 200-fold higher concentration of MTX was

required to cause growth inhibition of MDA-MB-468 cells equivalent in magnitude to that produced by ^{111}In-DTPA-hEGF. Paclitaxel, the most active chemotherapeutic agent, required almost a 100-fold higher concentration than ^{111}In-DTPA-hEGF to produce the same level of growth inhibition.

The antiproliferative effects of doxorubicin and paclitaxel were evaluated in combination with ^{111}In-DTPA-hEGF (Fig. 2). The growth rate of MDA-MB-468 cells was decreased 40% by incubation with 17 pM (<0.1 μCi/mL) of ^{111}In-DTPA-hEGF, but was decreased more than 90% when the cells were treated with ^{111}In-DTPA-hEGF for 5 days followed by treatment with 100 nM of doxorubicin for 2 days (Fig. 2 A). Treatment with 50 nM doxorubicin alone decreased the growth rate of the cells 4-fold. This effect was less pronounced with paclitaxel (Fig. 2 B). Treatment of MDA-MB-468 cells with 21 pM (<0.1 μCi/mL) of ^{111}In-DTPA-hEGF decreased the growth rate of the cells 2-fold, but in combination with 20 nM paclitaxel, cell growth was inhibited 3-4 fold. Treatment of MDA-MB-468 cells with 20 nM of paclitaxel alone resulted in a 1.5-fold decrease in the growth rate of the cells. These results suggest that the antiproliferative effects of targeted Auger electron radiotherapy using ^{111}In-DTPA-hEGF are additive with those of doxorubicin or paclitaxel.

Treatment of Breast Cancer Cells with External γ-Radiation. External γ-radiation decreased the growth of MDA-MB-468 cells 2-fold (ED_{50}) at a radiation absorbed dose of 4 Gy and 10-fold (ED_{90}) at a radiation absorbed dose of 6 Gy (Table 1). The antiproliferative effect on MDA-MB-468 cells was increased by combining γ-radiation and ^{111}In-DTPA-hEGF (Fig. 3). The growth of MDA-MB-468 cells was inhibited 2-fold by treatment with 4 Gy of γ-radiation, but the growth rate was decreased 16-fold when the cells were treated with 4 Gy of γ-radiation followed by 100 pM (0.5 μCi/mL) of ^{111}In-DTPA-hEGF for 6 days. Treatment of MDA-MB-468 cells with 100 pM (0.5 μCi/mL) of ^{111}In-DTPA-hEGF alone inhibited the growth of the cells 8-fold. These results suggest

that the antiproliferative effects of ^{111}In-DTPA-hEGF on MDA-MB-468 cells are additive with those produced by γ-radiation. Since the antiproliferative effect of 70 pM (0.34 μCi/mL) was similar to that produced by 4 Gy of external γ-radiation, these results further suggest that the two treatments resulted in equivalent radiobiological damage to radiation-sensitive targets in the cell.

Evaluation of Apoptosis and Necrosis. The effect of treatment of MDA-MB-468 breast cancer cells with ^{111}In-DTPA-hEGF or unlabeled hEGF on induction of apoptosis and necrosis of the cells was evaluated by flow cytometry using fluorescein-Annexin V or propidium iodide respectively (Fig. 4). The apoptotic fraction increased 70-fold following treatment for 4 days with unlabeled hEGF (200 pM) and more than 250-fold following treatment with ^{111}In-DTPA-hEGF (200 pM, 1 μCi/mL) (Fig.3). The proportion of necrotic cells increased 20-fold by treatment with unlabeled hEGF and more than 60-fold following treatment with ^{111}In-DTPA-hEGF. Our results confirm previous reports (11) that unlabeled EGF induces apoptosis in MDA-MB-468 cells, but further demonstrate that the extent of apoptosis and necrosis is significantly enhanced following treatment with ^{111}In-labeled hEGF.

Cell Cycle Analysis. Treatment of MDA-MB-468 cells with ^{111}In-DTPA-hEGF (250 pM, 1 μCi/mL) doubled the proportion of cells in G2/M phase compared to control, untreated cells, whereas treatment with unlabeled hEGF (250 pM) increased the proportion of cells in G2/M phase by only 25% (Fig. 5). The proportion of cells in S-phase remained unchanged but the proportion of cells in G1-phase decreased 15-20% in response to treatment with ^{111}In-DTPA-hEGF or unlabeled hEGF. External γ-radiation (10 Gy) caused mostly G2/M arrest (results not shown).

DNA Fragmentation. DNA fragmentation in MDA-MB-468 breast cancer cells treated with ^{111}In-DTPA-hEGF (80 pM, 0.4 μCi/mL) unlabeled hEGF (80 pM) or external γ-radiation (10 Gy) was evaluated using a "Comet" assay. All treatments caused DNA fragmentation as evidenced by the appearance of a Comet "tail". The extent of DNA fragmentation was qualitatively similar for treatment with ^{111}In-DTPA-hEGF or 10 Gy of external γ-radiation (Fig. 6), but was less for treatment with unlabeled hEGF (results not shown).

Discussion

To our knowledge, the results of this study reveal for the first time that Auger electron radiopharmaceuticals are significantly more potent on a molar concentration basis than chemotherapy in inhibiting the growth of EGFR-positive human breast cancer cells *in vitro* (Fig. 1 and Table 1). ^{111}In-DTPA-hEGF was almost 100-fold more potent at inhibiting the growth of MDA-MB-468 cells *in vitro* than paclitaxel (IC_{50} 70 pM vs 6 nM respectively) and the radiopharmaceutical was 300-400 fold more effective than doxorubicin, camptothecin or MTX (IC_{50} values of 15-30 nM). There was more than a 4-logarithm difference in antiproliferative potency between ^{111}In-DTPA-hEGF (IC_{50} 70 pM) and 5-FU (IC_{50} 4 μM). The IC_{50} values for paclitaxel, camptothecin and doxorubicin determined in our study were similar to those previously reported for MDA-MB-468 cells (12-14).

The strong antiproliferative effect of ^{111}In-DTPA-hEGF on MDA-MB-468 cells (IC_{50} 70 pM, 0.3 μCi/mL) was likely due to the DNA-damaging effects of the emitted Auger electrons. Although unlabeled hEGF was also growth inhibitory to MDA-MB-468 cells, it was much less potent (IC_{50} >200 pM). The antiproliferative effect of unlabeled hEGF on MDA-MB-468 cells may be due to the previously reported growth inhibitory properties of high concentrations (>1 nM) of

EGF on this cell line (15,16). Growth inhibition of MDA-MB-468 cells by ^{111}In-DTPA-hEGF may be mediated by cell cycle arrest and induction of apoptosis or necrosis. The apoptotic fraction of MDA-MB-468 cells exposed to ^{111}In-DTPA-hEGF was increased 250-fold compared to untreated cells and the proportion of necrotic cells was increased 60-fold (Fig. 3). Since the assays were conducted only on adherent cells, the results may have underestimated the true extent of apoptosis or necrosis. Cells killed by treatment with ^{111}In-DTPA-hEGF or unlabeled hEGF detached from the culture dishes and were not evaluated. Programmed cell death may be induced in cancer cells with irreversibly damaged chromosomal DNA (17). DNA fragmentation was clearly evident in MDA-MB-468 cells treated with 80 pM (0.4 μCi/mL) of ^{111}In-DTPA-hEGF using a "Comet" assay (Fig. 6). DNA fragmentation was also observed in MDA-MB-468 cells treated with unlabeled hEGF, possibly due to apoptotic DNA cleavage (15,18). Control, untreated MDA-MB-468 cells did not exhibit a significant Comet tail indicating that DNA fragmentation was not an artifact of the analysis technique.

The proportion of MDA-MB-468 cells in G2/M-phase was increased in response to treatment with ^{111}In-DTPA-hEGF (Fig. 5) or external γ-radiation (results not shown). MDA-MB-468 cells have a mutated p53 gene and do not arrest in G1-phase in response to radiation, due to the inability of the mutant p53 protein to transcriptionally upregulate the cyclin-dependent kinase inhibitor, $p21^{waf1/cip1}$ (19). High concentrations of EGF (>1 nM) can upregulate $p21^{waf1/cip1}$ by a p53-independent mechanism resulting in G1 arrest (20). At the low concentrations of ^{111}In-DTPA-hEGF (12.5-250 pM) evaluated in this study, we did not observe an increase in the proportion of MDA-MB-468 cells in G1-phase, or an increase in $p21^{waf1/cip1}$ expression by Western blot (results not shown). Targeted Auger electron radiotherapy appears therefore to have a similar effect on cell cycle kinetics in MDA-MB-468 cells as that produced by external γ-radiation.

It is generally not well appreciated that radiopharmaceuticals exert their cytotoxic effects at extremely low concentrations (picomolar range) since their mode of action requires the infliction of a single lethal insult to chromosomal DNA (ie. double-strand break). DNA damage caused by Auger electron-emitting radiopharmaceuticals is highly dependent on the proximity of the location of radionuclide decay to chromosomal DNA. We previously determined (5) that ^{111}In-DTPA-hEGF was rapidly internalized by MDA-MB-468 cells with up to 15% of the radiopharmaceutical localizing in the cell nucleus and 10% binding directly to chromatin. At the IC_{50} of 70 pM for ^{111}In-DTPA-hEGF, approximately 5% of EGFR on MDA-MB-468 cells would be occupied by radioligand assuming a K_a of 7.5×10^8 L/mol for the radiopharmaceutical (7). For a single MDA-MB-468 breast cancer cell expressing 1×10^6 receptors/cell, as many as 5×10^4 molecules of ligand would be bound by each cell and 10% of these (~5,000 molecules) would translocate to the cell nucleus and bind to chromosomal DNA. At the specific activity of ^{111}In-DTPA-hEGF (800 μCi/μg, 4.8×10^6 μCi/μmol) used in our study, approximately 1 in 10 hEGF molecules carry an ^{111}In atom.

Karamychev et al. (21) have calculated that 0.38 DNA breaks occurred for each ^{111}In decay in a 42-mer target DNA sequence when hybridized to an ^{111}In-labeled oligodeoxyribonucleotide. Assuming a similar frequency of DNA cleavage for ^{111}In-DTPA-hEGF bound to chromosomal DNA, approximately 190 DNA strand breaks would be anticipated in a MDA-MB-468 cell from the decay of 500 chromatin-bound ^{111}In atoms. Although most DNA strand breaks are repaired by the cell, any unrepaired breaks or those repaired incorrectly may be lethal. Thus, it seems probable that even at picomolar concentrations of ^{111}In-DTPA-hEGF, there would be sufficient DNA lesions remaining in a MDA-MB-468 breast cancer cell to result in cell death. This would also be consistent with the strong antiproliferative effect observed for ^{111}In-DTPA-hEGF on MDA-MB-468 cells at these concentrations. In contrast, the cytotoxicity of the chemotherapeutic agents tested

(paclitaxel, doxorubicin, camptothecin, MTX and 5-FU) depends on disruption of a biochemical or biological pathway in the cell leading to interference with protein or DNA synthesis (22-24). As demonstrated in our study and by others (12-14), modulation of these processes in breast cancer cells requires much higher (nanomolar or micromolar) concentrations of cytotoxic agents.

The results of this study revealed the higher potency of radiopharmaceuticals labeled with low energy, short-range Auger electron-emitting radionuclides compared to chemotherapeutic agents for treatment of EGFR-overexpressing breast cancer cells *in vitro*. We have also found a greater antitumor effect *in vivo* of ^{111}In-DTPA-hEGF compared to doxorubicin in nude mice implanted with subcutaneous MDA-MB-468 breast cancer xenografts (manuscript in preparation). Mice treated with 2.8 mCi of ^{111}In-DTPA-hEGF exhibited almost complete tumor growth arrest, whereas treatment with doxorubicin (20 mg/m^2/week) caused only tumor growth inhibition. There is also evidence that higher energy, longer-range β-emitting radiopharmaceuticals may be more effective than chemotherapy for treatment of tumors *in vivo*. DeNardo et al. (25) evaluated the efficacy of ^{90}Y-ChL6 mAb (RIT) alone or in combination with paclitaxel for treatment of HBT3477 breast cancer xenografts implanted in nude mice. No tumor responses were observed in mice treated with paclitaxel, but RIT produced tumor remissions in 23/29 animals. Similarly, Clarke et al. (26) recently found no effect of treatment with paclitaxel on growth of MCF-7 breast cancer xenografts in nude mice, whereas ^{131}I-3S193 anti-Le^y mAb resulted in tumor remissions in 5/5 animals. Superior antitumor effects have also been reported for RIT of colon cancer xenografts compared to 5-FU (27,28) and for RIT of medullary thyroid carcinoma xenografts compared to doxorubicin (29).

Notwithstanding promising results in animal tumor models, the results of clinical trials of radiopharmaceutical therapy of solid tumours including breast cancer have largely been disappointing. The reasons for inadequate tumor responses in humans in the case of RIT, relate

mostly to low tumor uptake of the radiopharmaceuticals combined with dose-limiting non-specific myelotoxicity due to the long range (several mm) of the β-particles (3). Radiopharmaceutical delivery to breast cancer cells is also a key issue which will ultimately determine the efficacy of targeted Auger electron radiotherapy of breast cancer using ^{111}In-DTPA-hEGF in humans, but myelotoxicity may not be a major concern, since the cytotoxicity of the radiopharmaceutical is restricted to cells which specifically bind and internalize the radiopharmaceutical, and <3% of bone marrow stem cells express EGFR (30). Encouragingly, there have been reports of objective tumor responses to RIT in breast cancer patients previously found to be refractory to chemotherapy in clinical trials, particularly where bone marrow toxicity was minimized by providing stem cell support (31,32).

Combining radiopharmaceutical treatment with chemotherapy or external γ-radiation is also an appealing strategy for treatment of breast cancer, since the disease is sensitive to both radiation and cytotoxic cancer therapy. In this study, treatment of MDA-MB-468 cells *in vitro* with ^{111}In-DTPA-hEGF followed by doxorubicin or paclitaxel increased the antiproliferative effect 2-5 fold (Fig. 2). An 8-fold enhanced growth inhibition of MDA-MB-468 cells was obtained by combining external γ-radiation followed by ^{111}In-DTPA-hEGF (Fig. 3). Similar results have been demonstrated *in vivo* by DeNardo et al. (25) who achieved complete cure of HBT3477 human breast cancer xenografts in almost half of mice treated with a combination of ^{90}Y-ChL6 mAb and paclitaxel, whereas ^{90}Y-ChL6 treatment alone yielded only tumor growth inhibition. An enhanced antitumor effect was also observed by Clarke et al. (26) in mice bearing MCF-7 human breast cancer xenografts using a combination of ^{131}I-3S193 mAb and paclitaxel. The results reported in this study further suggest that combining targeted Auger electron radiotherapy with conventional treatment

modalities (chemotherapy or external γ-radiation) may be a an effective treatment strategy for EGFR-overexpressing advanced breast cancer.

Conclusion

We conclude that targeted Auger electron radiotherapy using ^{111}In-DTPA-hEGF is a highly promising novel anticancer treatment for EGFR-overexpressing advanced breast cancer. Strong antiproliferative effects were achieved *in vitro* with the radiopharmaceutical on EGFR-overexpressing breast cancer cells at concentrations several logarithms lower than that required for chemotherapeutic agents. The growth inhibitory properties of ^{111}In-DTPA-hEGF on breast cancer cells were equivalent to those produced by several Gy of external γ-radiation. Cell growth inhibition was significantly enhanced by combining ^{111}In-DTPA-hEGF with chemotherapy or external γ-radiation. The antiproliferative effects of ^{111}In-DTPA-hEGF were mediated by DNA damage, cell cycle arrest in G2/M-phase and induction of apoptosis or necrosis. Studies are currently in progress to evaluate the antitumor effects *in vivo* of ^{111}In-DTPA-hEGF on the growth of EGFR-overexpressing human breast xenografts in athymic mice.

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References

1. Krenning, E. P., Valkema, R., Kooij, P. P. M., Breeman, W. A. P., Bakker, W. H., deHerder, W. W., vanEijck, C. H. J., Kwekkeboom, D. J., DeJong, M., and Pauwels, S. Peptide receptor radionuclide therapy with [indium-111-DTPA-D-Phe]-octreotide. J. Nucl. Med., *38*: 47P, 1997.

2. Mariani, G., Bodei, L., Adelstein, S. J., and Kassis, A. I. Emerging roles for radiometabolic therapy of tumors based on Auger electron emission. J. Nucl. Med., *41*: 1519-1521, 2000.

3. Reilly, R. M., Sandhu, J., Alvarez-Diez, T., Gallinger S., Kirsh J. and Stern HS Problems of delivery of monoclonal antibodies: Pharmaceutical and pharmacokinetic solutions. Clin. Pharmacokinet., *28*: 126-142, 1995.

4. Klijn, J. G. M., Berns, P. M. J. J., Schmitz, P. I. M., and Foekens, J. A. The clinical significance of epidermal growth factor receptor (EGF-R) in human breast cancer: a review on 5232 patients. Endocr. Rev., *13*: 3-17, 1992.

5. Reilly, R. M., Kiarash, R., Cameron, R. G., Porlier, N., Sandhu, J., Hill, R. P., Vallis, K., Hendler, A., and Gariépy, J. Indium-111 labeled EGF is selectively radiotoxic to human breast cancer cells overexpressing EGFR. J. Nucl. Med., *41*: 429-438, 2000.

6. Filmus, J., Trent, J. M., Pollak, M. N., and Buick, R. N. Epidermal growth factor receptor gene-amplified MDA-468 breast cancer cell line and its non-amplified variants. Molec. Cellular Biol., *7*: 251-257, 1987.

7. Reilly, R. M., Kiarash, R., Sandhu, J., Lee, Y. W., Cameron, R., Hendler, A., Vallis, K., and Gariépy, J. A comparison of epidermal growth factor and monoclonal antibody 528 labeled with indium-111 for imaging human breast cancer. J. Nucl. Med., *41*: 903-912, 2000.

8. Vermes, I., Haanen, C., Steffens-Nakken, H., and Reutelingsperger, C. Flow cytometric detection of phoshatidylserine expression on early apoptotic cells using fluorescein labelled annexin V. J. Immunol. Meth., *184*: 39-51, 1995.

9. ModFit LT. Ver. 2.0. 1996. Topsham, ME, Verity Software.

10. Fairbairn, D. W., Olive, P. L., and O'Neill, K. L. The comet assay: a comprehensive review. Mutation Res., *339*: 37-59, 1995.

11. Schaerli, P. and Jaggi, R. EGF-induced programmed cell death of human mammary carcinoma MDA-MB-468 cells is preceded by activation of AP-1. Cellular and Molecular Life Sci., *54*: 129-138, 1998.

12. McCloskey, D. E., Kaufmann, S. H., Prestigiacomo, L. J., and Davidson, N. E. Paclitaxel induces programmed cell death in MDA-MB-468 human breast cancer cells. Clin. Cancer Res., *2*: 847-854, 1996.

13. Liu, W. and Zhang, R. Upregulation of $p21^{WAF1/CIP1}$ in human breast cancer cell lines MCF-7 and MDA-MB-468 undergoing apoptosis induced by natural product anticancer drugs 10-hydroxycamptothecin and camptothecin through p53-dependent and independent pathways. Int. J. Oncol., *12*: 793-804, 1998.

14. Kratz, F., Beyer, U., Roth, T., Tarasova, N., Collery, P., Lechenault, F., Cazabat, A., Schumacher, P., Unger, C., and Falken, U. Transferrin conjugates of doxorubicin: synthesis, characterization, cellular uptake, and in vitro efficacy. J. Pharm. Sci., *87*: 338-346, 1998.

15. Armstrong, D.K., Kaufmann, S.H., Ottaviano, Y.L., Furuya, Y., Buckley, J.A., Isaacs, J.T. and Davidson, N.E. Epidermal growth factor-mediated apoptosis of MDA-MB-468 human breast cancer cells. Cancer Res., *54*: 5280-5283, 1994.

16. Filmus, J., Pollak, M. N., Cailleau, R., and Buick, R. N. A human breast cancer cell line with a high number of epidermal growth factor (EGF) receptors, has an amplified EGF receptor gene and is growth inhibited by EGF. Biochem. Biophys. Res. Commun., *128*: 898-905, 1985.

17. Kerr, J. F., Winterford, C. M., and Harmon, B. V. Apoptosis: its significance in cancer and cancer therapy. Cancer, *73*: 2013-2026, 1994.

18. Kottke, T. J., Blajeski, A. L., Martins, M., Mesner Jr., P. W., Davidson, N. E., Earnshaw, W. C., Armstrong, D. K., and Kaufmann, S. H. Comparison of paclitaxel-, 5-fluoro-2'-deoxyuridine-and epidermal growth factor (EGF)-induced apoptosis. Evidence for EGF-induced anoikis. J. Biol. Chem., *274*: 15927-15936, 1999.

19. El-Deiry, W. S., Harper, J. W., O'Connor, P. M., Velculescu, V. E., Canman, C.E., Jackman, J., Pietenpol, J.A., Burrell, M., Hill, D.E., Wang, Y., Wiman, K.G., Mercer, W.E., Kastan, M.B., Kohn, K.W., Elledge, S.J., Kinzler, K.W. and Vogelstein, B. WAF1/CIP1 is induced in p53-mediated G_1 arrest and apoptosis. Cancer Res., *54*: 1169-1174, 1994.

20. Thomas, T., Balabhadrapathruni, S., Gardner, C. R., Hong, J., Faaland, C. A., and Thomas, T. J. Effects of epidermal growth factor on MDA-MB-468 breast cancer cells: alterations in polyamine biosynthesis and the expression of p21/CIP1/WAF1. J. Cellular Physiol., *179*: 257-266, 1999.

21. Karamychev, V. N., Panyutin, I. G., Kim, M.-K., Le, N., Paik, C. H., Carrasquillo, J. A., Reed, M. W., and Neumann, R. D. DNA cleavage by ^{111}In-labeled oligodeoxyribonucleotides. J. Nucl. Med., *41*: 1093-1101, 2000.

22. Moore, M. J. and Erlichman, C. Pharmacology of anticancer drugs. *In:* I. F. Tannock and R. P. Hill (eds.), The Basic Science of Oncology. 3rd ed, pp. 370-391. Toronto: McGraw-Hill, 1998.

23. Tritton, T. R. and Yee, G. The anticancer agent adriamycin can be actively cytotoxic without entering cells. Science, *217*: 248-250, 1982.

24. Pommier, Y. Eukaryotic DNA topoisomerase I: genome gate keeper and its intruders, camptothecins. Semin. Oncol., *23*: 1-10, 1996.

25. DeNardo, S. J., Kukis, D. L., Kroger, L. A., O'Donnell, R. T., Lamborn, K. R., Miers, L. A., DeNardo, D. G., Meares, C. F., and DeNardo, G. L. Synergy of taxol and radioimmunotherapy with yttrium-90-labeled chimeric L6 antibody: efficacy and toxicity in breast cancer xenografts. Proc. Natl. Acad. Sci. USA, *94*: 4000-4004, 1997.

26. Clarke, K., Lee F-T., Brechbiel, M. W., Smyth, F. E., Old, L. J., and Scott, A. M. Therapeutic efficacy of anti-Lewis^{y} humanized 3S193 radioimmunotherapy in a breast cancer model: Enhanced activity when combined with taxol. Clin. Cancer Res., *6*: 3621-3628, 2000.

27. Tschmelitsch, J., Barendswaard, E., Williams Jr., C., Yao, T-J., Cohen, A.M., Old, L.J. and Welt, S. Enhanced antitumor activity of combination radioimmunotherapy (^{131}I-labeled monoclonal antibody A33) with chemotherapy (fluorouracil). Cancer Res. *57*: 2181-2186, 1997.

28. Blumenthal, R. D., Sharkey, R. M., Natale, A. M., Kashi, R., Wong, G., and Goldenberg, D. M. Comparison of equitoxic radioimmunotherapy and chemotherapy in the treatment of human colonic cancer xenografts. Cancer Res., *54*: 142-151, 1994.

29. Stein, F., Juweid, M., Zhang, C.-H., and Goldenberg, D. M. Assessment of combined radioimmunotherapy and chemotherapy for treatment of medullary thyroid cancer. Clin. Cancer Res., *5*: 3199s-3206s, 1999.

30. Waltz, T. M., Malm, C., Nishikawa, B. K., and Wasteson, A. Transforming growth factor-alpha (TGF-alpha) in human bone marrow: demonstration of TGF-alpha in erythroblasts and eosinophilic precursor cells and of epidermal growth factor receptors in blastlike cells of myelomonocytic origin. Blood, *85*: 2385-2392, 1995.

31. Schrier, D. M., Stemmer, S. M., Johnson, T., Kasliwal, R., Lear, J., Matthes, S., Taffs, S., Dufton, C., Glenn, S. D., Butchko, G., and et al. High-dose ^{90}Y Mx-diethylenetriaminepentaacetic acid (DTPA)-BrE-3 and autologous hematopoietic stem cell support (AHSCS) for treatment of advanced breast cancer: a phase I trial. Cancer Res., *55*: 5921s-5924s, 1995.

32. DeNardo, S. J., O'Grady, L. F., Richman, C. M., Goldstein, D. S., O'Donnell, R. T., DeNardo, D. A., Kroger, L. A., Lamborn, K. R., Hellstrom, I., and DeNardo, G. L. Radioimmunotherapy for advanced breast cancer using I-131-ChL6 antibody. Anticancer Res., *17*: 1745-1751, 1997.

Table 1. *Inhibition of the growth of MDA-MB-468 human breast cancer cells treated in vitro with ^{111}In-DTPA-hEGF, unlabeled hEGF, chemotherapeutic agents or external γ-radiation.*

MDA-MB-468 cells were treated *in vitro* with ^{111}In-DTPA-hEGF, unlabeled hEGF, chemotherapeutic agents or external γ-radiation and the growth of the cells compared to untreated cells over 7-days was measured colorimetrically as described in "Materials and Methods".

Treatment	Range Studied	IC_{50} or ED_{50}	IC_{90} or ED_{90}
^{111}In-DTPA-hEGF	12.5-200 pM	70 pM	200 pM
Unlabeled hEGF	12.5-200 pM	>200 pM	n.d.
Paclitaxel	0.8-25 nM	6 nM	20 nM
MTX	15-120 nM	15 nM	70 nM
Doxorubicin	3-200 nM	20 nM	75 nM
Camptothecin	3-100 nM	30 nM	75 nM
5-FU	1-20 μM	4 μM	>10 μM
External γ-Radiation	2-20 Gy	4 Gy	6 Gy

n.d.: Not determined.

Legends for Figures

Fig. 1. Growth inhibition of MDA-MB-468 human breast cancer cells produced by increasing concentrations of ^{111}In-DTPA-hEGF (12.5-200 pM, 0.02-1 μCi/mL) or chemotherapeutic agents: doxorubicin, camptothecin or paclitaxel. MDA-MB-468 cells were treated with the radiopharmaceutical or chemotherapeutic agents and the number of viable cells at the end of a 7-day culture period measured colorimetrically as described in "Materials and Methods". The percent growth inhibition is expressed relative to untreated cells. The IC_{50} for ^{111}In-DTPA-hEGF (70 pM, 0.3 μCi/mL) was 100-400 fold lower than that for chemotherapeutic agents (IC_{50} 6-30 nM).

Fig. 2. Growth inhibition of MDA-MB-468 human breast cancer cells by treatment with 17 pM (0.1 μCi/mL) of ^{111}In-DTPA-hEGF combined with increasing concentrations of doxorubicin (A) or paclitaxel (B). Cells were cultured with the radiopharmaceutical for 5 days followed by treatment with the chemotherapeutic agent for 2 days and the number of viable cells was determined colorimetrically as described in "Materials and Methods". The percent growth inhibition is expressed relative to untreated cells. The percent growth inhibition was increased 2-5 fold using a combination of ^{111}In-DTPA-hEGF treatment and chemotherapy.

Fig. 3. Growth inhibition of MDA-MB-468 human breast cancer cells by treatment with 100 pM (0.5 μCi/mL) of ^{111}In-DTPA-hEGF combined with increasing doses of external γ-radiation. Cells were treated with external γ-radiation using a ^{137}Cs source, then cultured in the presence of the radiopharmaceutical for 6 days. The number of viable cells was determined colorimetrically as described in "Materials and Methods". The percent growth inhibition is expressed relative to

untreated cells. The percent growth inhibition was increased 8-fold using a combination of ^{111}In-DTPA-hEGF treatment and external γ-radiation.

Fig. 4. Flow cytometric analysis of adherent MDA-MB-468 cells stained with fluorescein-Annexin V or propidium iodide. The cells received no treatment (A) or were treated with unlabeled hEGF (200 pM) (B) or ^{111}In-DTPA-hEGF (200 pM, 1 μCi/mL) (C) as described in "Materials and Methods". Apoptotic cells are displayed in the lower right quadrant and necrotic cells in the upper right quadrant. ^{111}In-DTPA-hEGF treatment increased the proportion of apoptotic cells 250-fold and increased the proportion of necrotic cells 60-fold compared to untreated cells. Unlabeled hEGF increased the proportion of apoptotic cells 70-fold and the proportion of necrotic cells 20-fold compared to untreated cells.

Fig. 5. Cell cycle analysis of MDA-MB-468 human breast cancer cells treated with 250 pM of unlabeled hEGF (A) or 250 pM (1 μCi/mL) of ^{111}In-DTPA-hEGF (B) for 4 days as described in "Materials and Methods". The curves were analysed using Mod-Fit LT software which displays the relative area under the curve for cells in G1-phase (white area), S-phase (cross-shaded area) or G2/M-phase (black area). The proportion of cells in G2/M-phase was doubled following treatment with ^{111}In-DTPA-hEGF but was increased only 25% by treatment with unlabeled hEGF.

Fig. 6. Electrophoretic analysis ("Comet" assay) of DNA isolated from single MDA-MB-468 cells receiving no treatment (A) or treated with 80 pM (0.4 μCi/mL) of ^{111}In-DTPA-hEGF (B) or 10 Gy of external γ-radiation (C) as described in "Materials and Methods". The extent of DNA damage

produced by Auger electrons emitted by the radiopharmaceutical was qualitatively similar to that produced by external γ-radiation.

Fig. 1

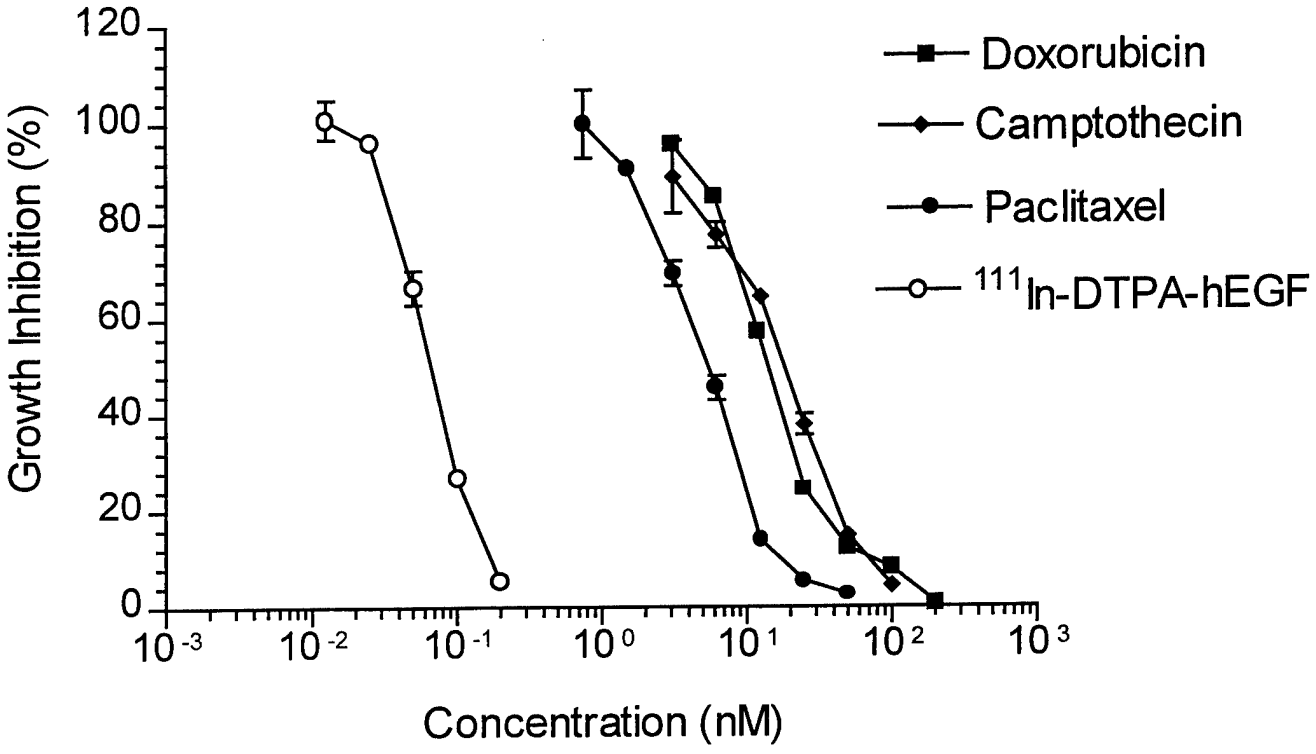

Growth Inhibition (%)
120
100
80
60
40
20
0
10 -3
10 -2
10 -1
10 0
10 1
10 2
10 3
Concentration (nM)
Doxorubicin
Camptothecin
Paclitaxel
111In-DTPA-hEGF

Fig. 2.

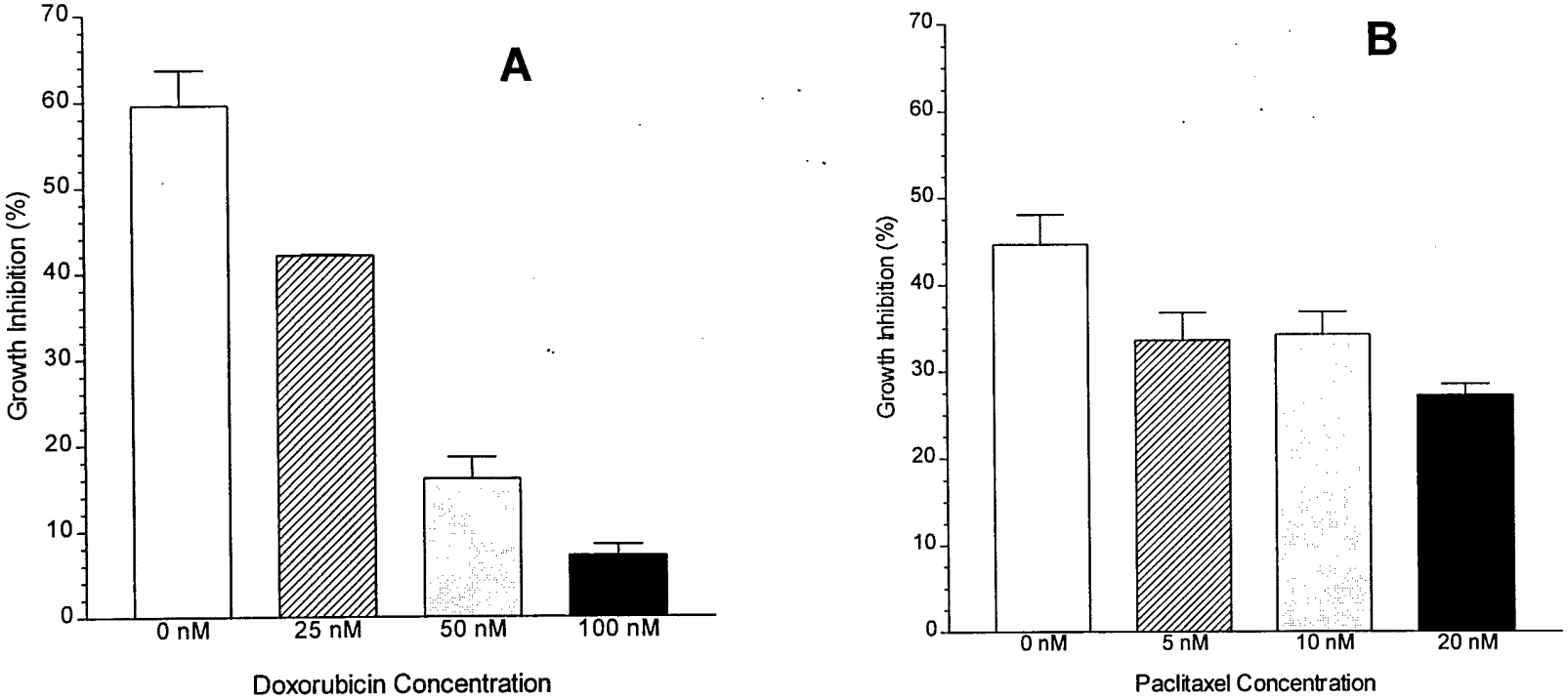

A
Growth Inhibition (%)
70
60
50
40
30
20
10
0
0 nM
25 nM
50 nM
100 nM
Doxorubicin Concentration
B
Growth Inhibition (%)
70
60
50
40
30
20
10
0
0 nM
5 nM
10 nM
20 nM
Paclitaxel Concentration

Fig. 3

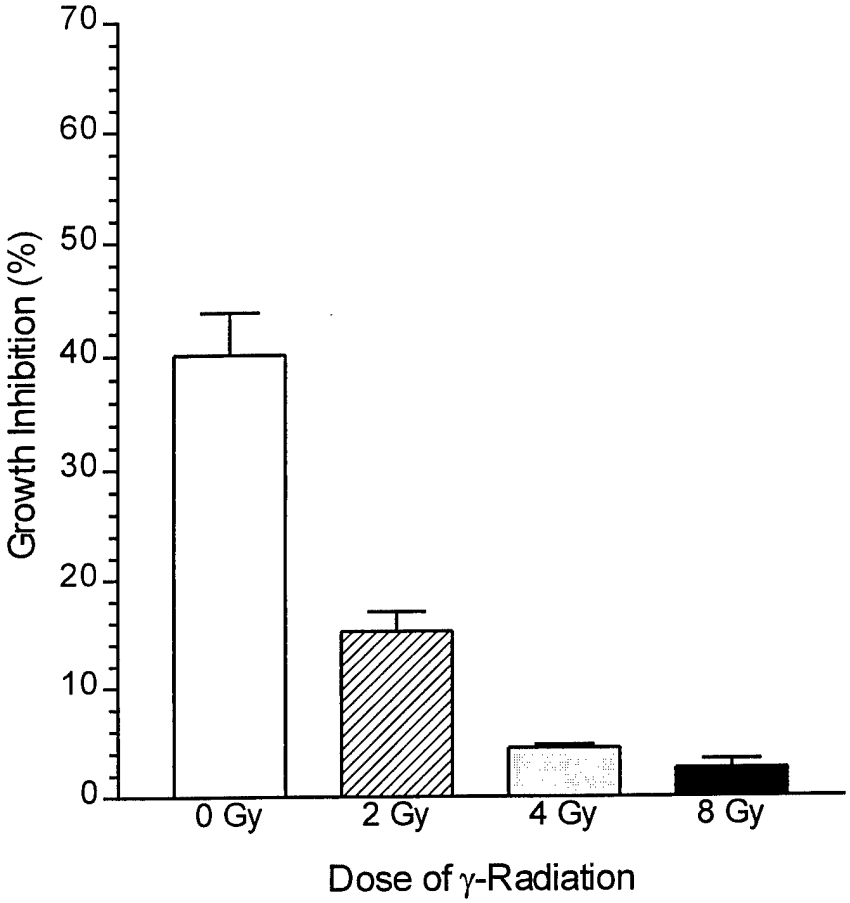

70
60
50
40
30
20
10
0
Growth Inhibition (%)
0 Gy
2 Gy
4 Gy
8 Gy
Dose of γ-Radiation

Figure 4

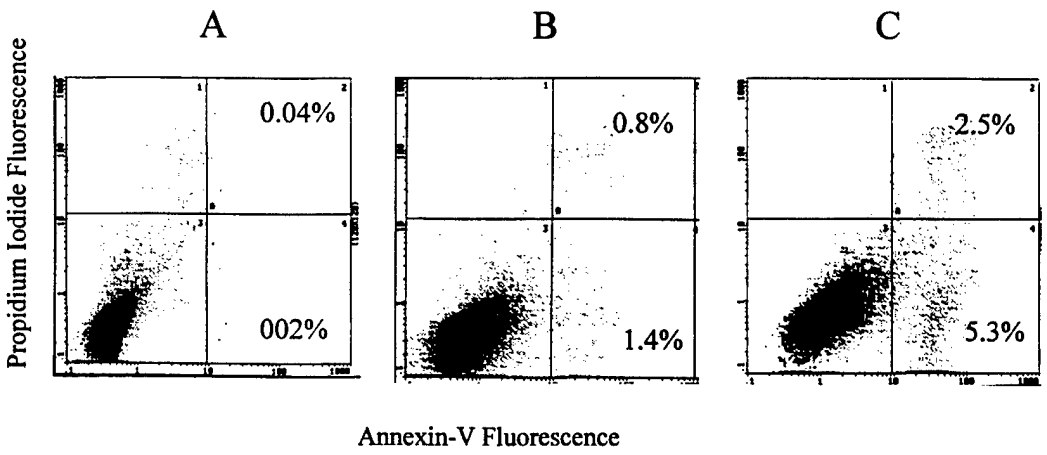
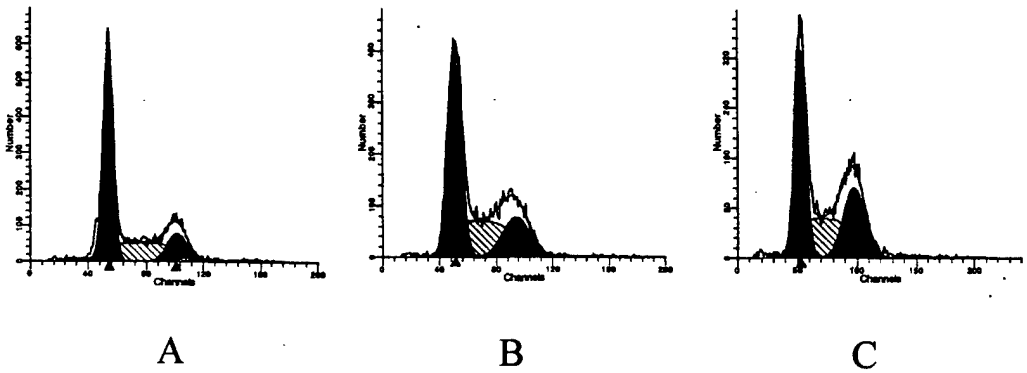


Figure 5



Nucl Med Biol 2001 (submitted)

AMPLIFIED DELIVERY OF INDIUM-111 TO EGFR-POSITIVE HUMAN BREAST CANCER CELLS

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Abbreviated Title: Amplified delivery of indium-111 to breast cancer cells

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ABSTRACT

A method is described to amplify the delivery of ^{111}In to human breast cancer cells utilizing a novel human serum albumin-human EGF (HSA-hEGF) bioconjugate substituted preferentially in the HSA domain with multiple DTPA metal chelators for ^{111}In. ^{111}In-DTPA-HSA-hEGF exhibited a lower receptor-binding affinity than ^{111}In-DTPA-hEGF but was rapidly and specifically bound, internalized and translocated to the nucleus in EGFR-positive MDA-MB-468 breast cancer cells. ^{111}In-DTPA-HSA-hEGF was cytotoxic *in vitro* mainly through the emission of short-range Auger electrons and partially through the effects of the hEGF moiety to MDA-MB-468 cells overexpressing EGFR (1-2 × 10^6 receptors/cell) but not towards MCF-7 breast cancer cells with a 100-fold lower level of EGFR on their surface. The cytotoxicity *in vitro* against MDA-MB-468 cells of ^{111}In-DTPA-HSA-hEGF substituted with nine DTPA chelators was enhanced 4-fold compared to ^{111}In-DTPA-hEGF monosubstituted with DTPA. Studies are planned to further evaluate ^{111}In-DTPA-HSA-hEGF *in vivo* as a new imaging and targeted radiotherapeutic agent for breast cancer.

INTRODUCTION

The epidermal growth factor receptor (EGFR) is an important target for the development of novel agents for treatment of breast cancer [2] since it is overexpressed on the majority of estrogen receptor-negative, hormone-insensitive and poor prognosis forms of the disease [14]. Radiopharmaceuticals directed towards EGFR may allow non-invasive molecular imaging of breast cancer by characterizing tumour receptor status, and thereby predict response to novel therapeutic agents directed towards the receptor. We previously showed that human breast cancer xenografts overexpressing EGFR implanted subcutaneously in athymic mice could be imaged using anti-EGFR monoclonal antibody 528 (mAb 528) or human EGF (hEGF) labeled with ^{111}In [19]. Tumor accumulation was greatest for ^{111}In-mAb 528 resulting in higher tumor/blood ratios despite the more rapid blood clearance of ^{111}In-hEGF. Nevertheless, hEGF has advantages over mAb 528 in that its rapid internalization and nuclear translocation can be exploited to selectively insert ^{111}In into the cytoplasm and nucleus of EGFR-positive breast cancer cells, where the emitted Auger electrons are highly damaging to DNA, resulting in cell death [18]. Thus, ^{111}In-hEGF is a promising new radiopharmaceutical for imaging and targeted radiotherapy of EGFR-positive breast cancer.

Receptor-binding proteins are labeled with ^{111}In by introduction of the strong metal chelator DTPA into the protein, usually by reaction of the bicyclic anhydride of DTPA (cDTPAA) with an ε-amino group on a lysine residue or the α-amino group on the protein [11]. In the case of hEGF, there are two lysine residues (K_{28} and K_{48}) and the N-terminal asparagine residue which are potential sites for DTPA derivatization. In our experience, reaction of hEGF with a 10-fold molar excess of cDTPAA results in only monosubstitution of the peptide with DTPA, which limits the maximum theoretical specific activity for labeling with ^{111}In to $<1.8 \times$

10^6 MBq/μmol (approx. 300 MBq/μg). In practice, a specific activity no greater than 2.4×10^5 MBq/μmol (40 MBq/μg) has been achieved. At this relatively low specific activity, only about 1 in 8 molecules of hEGF carry an ^{111}In atom and almost 90% of receptors targeted by the radiopharmaceutical are therefore occupied by non-radioactive ligand. This could limit the sensitivity for imaging breast cancer and would restrict the maximum amount of ^{111}In which can be delivered to breast cancer cells for targeted radiotherapy of the disease.

In this report, we describe a method for amplified delivery of ^{111}In to EGFR-positive breast cancer cells utilizing a novel human serum albumin-hEGF bioconjugate (HSA-hEGF) multiply substituted with DTPA. The focus of the current communication is the construction and characterization of the ^{111}In-DTPA-HSA-hEGF bioconjugate with respect to its purity, receptor-binding and internalization as well as its ability to mediate selective cytotoxicity *in vitro* against human breast cancer cells overexpressing EGFR. In future studies, we plan to evaluate ^{111}In-DTPA-HSA-hEGF *in vivo* in athymic mice implanted subcutaneously with EGFR-positive breast cancer xenografts for imaging and targeted radiotherapy of the disease.

MATERIALS AND METHODS

Breast Cancer Cells

MDA-MB-468 and MCF-7 human breast cancer cells displaying approx. $1\text{-}2 \times 10^6$ EGFR/cell [5] or 1×10^4 EGFR/cell respectively were purchased from ATCC (Manassas, VA). MDA-MB-468 cells were cultured in DMEM (Sigma, St. Louis, MO) supplemented with 10% fetal calf serum (FCS) and MCF-7 cells were cultured in α-MEM (Sigma) supplemented with 10% FCS.

Construction and Characterization of Human Serum Albumin-hEGF

HSA-hEGF was constructed by reaction of maleimide-derivatized hEGF with thiolated HSA (Fig. 1). Maleimide groups were introduced into hEGF (Upstate Biotechnology, Lake Placid, NY) by reaction of a 4 mg/mL solution of hEGF in phosphate-buffered saline, pH 7.3 (PBS) with a 10-fold molar excess of sulfo-SMCC (Pierce, Rockford, IL) at 37 °C for 30 mins. Thiol groups were introduced into HSA (Sigma) by reaction of HSA (10 mg/mL in 150 mM NaCl containing 50 mM triethanolamine and 1 mM disodium EDTA) with a 10-fold molar excess of 2-iminothiolane (Pierce) for 45 mins at room temperature under nitrogen. Meleimide-hEGF was purified on a P-2 mini-column (BioRad, Mississauga, ON) eluted with PBS, then concentrated to 5 mg/mL on a Centricon YM-3 ultrafiltration device (Amicon, Beverly, MA). Thiolated HSA was purified on a Sephadex G-25 mini-column (Pharmacia, Uppsula, Sweden) eluted with PBS containing disodium EDTA, then concentrated to 12 mg/mL on a Centricon YM-30 device. A 3-fold molar excess of maleimide-hEGF was reacted with thiolated HSA overnight at 4 °C. Monomeric HSA-hEGF was purified from polymerized species by passage through a Centricon YM-100 device and from excess hEGF by passage through a Centricon YM-30 device.

HSA-hEGF was analysed for purity and homogeneity by SDS-PAGE, Western blot and size-exclusion HPLC. SDS-PAGE was performed under non-reducing conditions on a 4-20% Tris HCL gradient mini-gel (BioRad) stained with Coomassie R-250 brilliant blue (BioRad). Western blot was conducted by transferring electrophoresed proteins onto a nitrocellulose membrane (Trans-Blot®, BioRad) and probing with an anti-HSA (Sigma) or anti-hEGF rabbit polyclonal antibody (provided by Dr. J. Gariépy, Ontario Cancer Institute). Bands were detected using a goat anti-rabbit antibody conjugated to horseradish peroxidase followed by incubation with diamidobenzidine chromogenic substrate and 0.03% H_2O_2. HPLC was performed on a

Progel TSK swxl G2000 column eluted with 100 mM KH_2PO_4/100 mM Na_2SO_4 pH 7.0 at a flow rate of 1 mL/min with UV detection at 280 nm. The hEGF substitition level (moles hEGF/mole HSA) was measured using the ChemiKine™ Human EGF EIA kit (Chemicon Inc., Ternecula, CA).

Radiolabeling of Human Serum Albumin-hEGF with ^{111}In

HSA-hEGF was derivatized with multiple DTPA metal chelators by reaction with a 10-fold to 100-fold molar excess of cDTPAA (Sigma) as previously described for hEGF [19]. The conjugation efficiency was measured by trace-labeling an aliquot of the reaction mixture with ^{111}In acetate and analysing the proportion of ^{111}In-DTPA-HSA-hEGF and free ^{111}In-DTPA by instant thin layer-silica gel chromatography (ITLC-SG, Gelman, Ann Arbor, MI) developed in 100 mM sodium citrate buffer pH 5.0. DTPA substitution (moles DTPA/mole HSA-hEGF) was calculated by multiplying the conjugation efficiency by the molar ratio of cDTPAA:HSA-hEGF used in the reaction. DTPA-HSA-hEGF was purified on a Sephadex G-50 mini-column eluted with 50 mM $NaHCO_3$ in 150 mM NaCl buffer pH 7.5 then reconcentrated to 10 mg/mL on a Centricon YM-30 device.

DTPA-HSA-hEGF was labeled with ^{111}In by incubation with ^{111}In acetate for 30 mins at room temperature. ^{111}In acetate was prepared by mixing equal volumes of trace-metal free 1 M sodium acetate buffer pH 6.0 and ^{111}In chloride (MDS-Nordion, Kanata, ON). For receptor-binding experiments, ^{111}In-DTPA-HSA-hEGF was labeled to a specific activity of 1-2 MBq/μg (5.5×10^4-1.1×10^5 MBq/μmole). For *in vitro* cytotoxicity studies, ^{111}In-DTPA-HSA-hEGF was labeled to a higher specific activity of 42 MBq/μg (2.7×10^6 MBq/μmol) and ^{111}In-DTPA-hEGF was labeled to 40 MBq/μg (2.4×10^5 MBq/μmol) as previously described [18]. The

radiochemical purity of ^{111}In-DTPA-HSA-hEGF and ^{111}In-DTPA-hEGF was >95% by ITLC-SG developed in 100 mM sodium citrate buffer pH 5.0.

Measurement of Receptor-Binding Properties

The receptor-binding properties of ^{111}In-DTPA-HSA-hEGF were evaluated in a direct radioligand binding assay using MDA-MB-468 human breast cancer cells (1-2 × 10^6 EGFR/cell). Briefly, ^{111}In-DTPA-HSA-hEGF (40 ng-5 μg) in 1 mL of 150 mM NaCl containing 0.2% bovine serum albumin was incubated with 3-5 × 10^6 cells in 1.5 mL microtubes with occasional mixing for 30 mins at 37 °C. Cell bound radioactivity was separated from free radioactivity by centrifugation at 2,700 × g for 5 mins, then counted in a γ-scintillation counter. Non-specific binding was determined by conducting the assay in the presence of an excess (100 nM) of unlabeled hEGF. Specific binding was obtained by subtraction of non-specific binding from total binding. The K_a and B_{max} values were estimated from a non-linear fitting of the specific binding versus the concentration of ^{111}In-DTPA-HSA-hEGF using GraphPad Prism software [15].

Determination of Internalization and Nuclear Translocation

Internalization and nuclear translocation was evaluated qualitatively by fluorescence microscopy by incubating a 100 nM solution of fluorescein-conjugated HSA-hEGF in 150 mM NaCl for 1 h at 37 °C with 1 × 10^4 MDA-MB-468 cells grown overnight on a chamber slide (Nunc, Life Technologies, Burlington, ON). The cells were washed with 150 mM NaCl and counterstained using the nuclear stain DAPI (Boehringer-Mannheim, Laval, PQ), then examined under a fluorescence microscope. In addition, cell fractionation studies were performed with ^{111}In-DTPA-HSA-hEGF to quantitate the amount of radioactivity internalized by MDA-MB-468 cells

and the amount of radioactivity imported into the cell nucleus. Briefly, 1×10^6 MDA-MB-468 cells in a 35 mm culture dish were incubated with 5 ng of ^{111}In-DTPA-HSA-hEGF for 30 mins at 37 °C. The cells were recovered from the dishes, washed with PBS and centrifuged at 960 × g for 5 mins to separate cell-bound radioactivity from free radioactivity. The proportion of internalized ^{111}In-DTPA-HSA-hEGF was determined by displacing cell-surface radioactivity with 200 mM sodium acetate/500 mM NaCl pH 2.5 at 4 °C. The proportion of radioactivity imported into the cell nucleus was determined by isolating the nuclei by lysing the cells in 10 mM Tris buffer pH 7.6 containing 350 mM sucrose, 10 mM KCl, 1.5 mM $MgCl_2$ and 0.2% Triton X-100 (BioRad) pH 7.6 under ultrasonication for 5 mins and centrifuging the lysate at 2,700 × g. This procedure has previously been found by us [18] and others [16] to result in intact nuclei without contamination by cytoplasmic organelles or fragments of cell membrane.

Determination of Cytotoxicity In Vitro

The selective cytotoxicity *in vitro* of ^{111}In-DTPA-HSA-hEGF for human breast cancer cells overexpressing EGFR was evaluated by dispensing 1×10^3 MDA-MB-468 ($1\text{-}2 \times 10^6$ receptors/cell) or MCF-7 cells (1×10^4 receptors/cell) into wells in a 96-well culture plate (Nunclon, Canadian Life Technologies, Burlington, ON), culturing the cells overnight, then treating the cells with increasing concentrations (7.5-250 pM) of ^{111}In-DTPA-HSA-hEGF or ^{111}In-DTPA-hEGF in growth medium for 7 days. ^{111}In-DTPA-HSA-hEGF contained nine DTPA groups per molecule and had a specific activity of 42 MBq/μg (2.7×10^6 MBq/μmol). ^{111}In-DTPA-hEGF was monosubstituted with DTPA and had a 10-fold lower specific activity (40 MBq/μg, 2.4×10^5 MBq/μmol). Control wells contained cells cultured in growth medium alone. Cell growth was determined colorimetrically using the WST-1 cell viability assay (Boehringer-

Mannheim) by measuring absorbance at 450 nm in a plate reader (Bio-Tek Model ELx800, Winnooski, VT). The concentration of ^{111}In-DTPA-HSA-hEGF or ^{111}In-DTPA-hEGF required to cause 50% growth inhibition (IC_{50}) was estimated from the growth inhibition curves.

Statistical Comparisons

Statistical comparisons were made using Student's t-test ($p<0.05$).

RESULTS AND DISCUSSION

A mostly monomeric HSA-hEGF bioconjugate was produced by reaction of maleimide-HSA with thiolated hEGF as evidenced by the appearance of one major band and a second less intense band on SDS-PAGE, corresponding to proteins with M_r of 62 kDa and 67 kDa respectively (Fig. 2). Only traces of polymerized species of higher molecular weight were observed by SDS-PAGE analysis. SDS-PAGE of unconjugated HSA or hEGF demonstrated one major band corresponding to proteins with the expected M_r of 57 kDa or 6 kDa respectively. Western blot (not shown) confirmed that HSA-hEGF contained both hEGF and HSA moieties. Both the 62 kDa and 67 kDa bands were positive by Western blot for HSA and hEGF moieties, but the most intense band was the 62 kDa band. The Western blot results suggest mostly monosubstitution of HSA with hEGF with a small proportion (<10%) of disubstituted bioconjugate. Size-exclusion HPLC of HSA-hEGF (Fig. 3) showed one major peak with retention time (t_R) of 11.8 mins and a second peak with t_R of 10.5 mins. A few additional minor peaks were also observed (including a peak with t_R of 16.0 mins corresponding to unconjugated hEGF), but these accounted for <5% of the total protein concentration. HPLC analysis of unconjugated HSA (not shown) similarly demonstrated two predominant peaks but with slightly longer retention times (t_R of 12.1 mins

and 10.8 mins). The small decrease in retention time for the HPLC peaks associated with HSA following hEGF conjugation and the small increase in M_r by SDS-PAGE were in agreement with an hEGF EIA which indicated that the hEGF substitution level was 0.9 ± 0.1 hEGF molecules/molecule bioconjugate (n = 3).

The receptor-binding affinity for ^{111}In-DTPA-HSA-hEGF substituted with 1-2 DTPA groups/molecule (K_a 5.1 ± 1.3 × 10^7 L/mole, Table 1) was approx. 15-fold lower than that of similarly substituted ^{111}In-DTPA-hEGF (7.5 ± 3.8 × 10^8 L/mole) [19]. There was no significant incremental decrease in affinity however as the DTPA substitution level was increased up to 23 DTPA groups/molecule (K_a 3.4 ± 0.9 × 10^7 L/mole). The number of receptors recognized on MDA-MB-468 cells was not significantly different for ^{111}In-DTPA-HSA-hEGF (B_{max} 1.4-2 × 10^6 receptors/cell) or ^{111}In-DTPA-hEGF (B_{max} 1.3 × 10^6 receptors/cell) [19]. These results suggest that the diminished receptor-binding affinity was mainly due to conjugation of hEGF with HSA rather than derivatization with multiple DTPA metal chelators. This would also be consistent with preferential substitution of DTPA onto the HSA moiety, a region of the molecule remote from the receptor-binding domain. HSA contains 60 lysine residues, whereas hEGF contains only two lysines (K_{28} and K_{48}) which present ε-amino groups for reaction with cDTPAA [9]. Diminished receptor-binding affinity may be due to steric hindrence as a result of reaction of thiolated HSA with hEGF derivatized with maleimide at K_{48}, a residue proximal to the putative receptor-binding motif of hEGF [12]. Others have utilized murine EGF (mEGF) which does not contain lysine residues to link macromolecules site-specifically to the α-amino group of EGF [8, 10]. Although mEGF bioconjugates are reported to exhibit relatively preserved receptor-binding properties, xenogeneic growth factors are known to be immunogenic in humans and may prevent repeated administration for imaging or radiotherapeutic applications [1]. The

HSA-hEGF bioconjugate produced was predominantly monosubstituted with hEGF but there may be advantages in a disubstituted hEGF-HSA bioconjugate since this could theoretically increase receptor binding avidity in an analagous manner to that for divalent antibody binding to cell surface antigens. Cross-linking of cell surface EGFR through two hEGF moieties may also promote internalization of the receptor-bioconjugate complex [4].

HSA-hEGF was rapidly internalized and translocated to the nucleus in MDA-MB-468 cells. Fluorescence microscopy of MDA-MB-468 cells incubated with fluorescein-conjugated HSA-hEGF for 30 mins at 37 °C (Fig. 4) showed fluorescence on the cell surface, in the cytoplasm and surrounding the cell nucleus (identified by counterstaining with DAPI). Cell fractionation studies with ^{111}In-DTPA-HSA-hEGF further revealed that 31.1 ± 0.6% of radioactivity remained on the cell surface, 52.6 ± 1.6% was internalized into the cytoplasm and 16.3 ± 2.6% was imported into the cell nucleus within 30 mins at 37 °C. The proportion of internalized ^{111}In-DTPA-HSA-hEGF (approx. 70%) was very similar to that previously measured for ^{111}In-DTPA-hEGF (approx. 67%) [18], but the fraction of radioactivity imported into the nucleus was about two-fold higher for ^{111}In-DTPA-HSA-hEGF.

^{111}In-DTPA-HSA-hEGF and ^{111}In-DTPA-hEGF were selectively cytotoxic to MDA-MB-468 cells overexpressing EGFR (Fig. 5 A and B). The modest (up to 1.3-fold) growth stimulatory effects of the radiopharmaceuticals on MCF-7 cells may be explained by the mitogenicity of EGF on breast cancer cells with very low EGFR expression [6]. This is in contrast to the effects on breast cancer cells with a high number of EGFR on their surface, which are growth-inhibited by EGF [5]. Since EGF is growth-inhibitory to MDA-MB-468 cells at high concentrations, a small proportion of the cytotoxic effects of ^{111}In-DTPA-hEGF and ^{111}In-DTPA-hEGF, particularly at concentrations >50 pM may be due to the hEGF moiety as previously reported

[3,18]. ^{111}In-DTPA-HSA-hEGF was approx. 4-fold more cytotoxic to MDA-MB-468 cells than ^{111}In-DTPA-hEGF (IC_{50} of 15 pM versus 60 pM respectively). The IC_{50} for unlabeled hEGF on MDA-MB-468 cells is >200 pM (Fig. 5 C) [3] indicating that the antiproliferative effects of the radiopharmaceuticals were mediated mainly by the emission of Auger electrons from ^{111}In. The increase in cytotoxic potency for ^{111}In-DTPA-HSA-hEGF was lower than expected however, since the specific activity was increased 10-fold compared to ^{111}In-DTPA-hEGF. This may be due to the reduced receptor-binding affinity of ^{111}In-DTPA-HSA-hEGF which may decrease the amount of radioactivity targeted to the cells.

One potential limitation to the clinical use of ^{111}In-DTPA-HSA-hEGF is its 10-fold higher molecular weight compared to ^{111}In-DTPA-hEGF (M_r 62-67 kDa versus 6 kDa) which could decrease tumor penetration [13, 17]. However, monoclonal antibody Fab' fragments which have a similar molecular size (M_r 50 kDa) penetrate deeply into tumor nodules in human cancer xenograft models [21] and tumor vasculature is known to be "leaky" to macromolecules including plasma proteins such as serum albumin [20]. The lower receptor-binding affinity of ^{111}In-DTPA-HSA-hEGF compared to ^{111}In-DTPA-hEGF may facilitate tumor penetration by minimizing the effect of the "binding-site barrier" [7]. It has been theorized that a receptor binding affinity in the range of 5×10^7-1×10^8 L/mole may be optimal to ensure deep tumor penetration and uniformity of intratumoral distribution [7]. Accumulation in normal tissues which express moderate levels of EGFR such as the liver and kidneys is also an important issue. Our previous experience with ^{111}In-DTPA-hEGF has shown however that very high amounts of radioactivity (74 MBq/mouse equivalent to a human dose of 14,208 MBq/m^2) can be safely administered to mice without any detectable evidence of liver or renal toxicity [18]. This may be due to the quiescent nature of these tissues or to high levels of free-radical scavengers such as

glutathione. Future studies are planned to further evaluate the tumor and normal tissue accumulation of ^{111}In-DTPA-HSA-hEGF *in vivo* in athymic mice implanted subcutaneously with human breast cancer xenografts and the potential utility of the radiopharmaceutical as a novel imaging or radiotherapeutic agent for EGFR-positive breast cancer.

CONCLUSIONS

We have described a method for amplified delivery of ^{111}In to EGFR-positive human breast cancer cells utilizing a novel HSA-hEGF bioconjugate multiply substituted with DTPA and labeled to high specific activity with ^{111}In. ^{111}In-DTPA-HSA-hEGF was specifically bound, internalized and translocated to the nucleus in breast cancer cells. The internalization of the radiopharmaceutical resulted in selective and enhanced cytotoxicity *in vitro* to breast cancer cells overexpressing EGFR mainly through the emission of Auger electrons and partially through the antiproliferative effects of the hEGF moiety. The feasibility of such an amplification strategy has also been successfully demonstrated by others for delivering multiple chlorin-e_6 photosensitizers to EGFR-positive breast cancer cells for photodynamic therapy [10] or boron hydride atoms to glioblastomas for boron neutron capture therapy [8]. In this communication, we extend the approach to the delivery of radionuclides to EGFR-positive breast cancer cells.

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References

1. L.O. Almer, G. Ekberg, S. Fankhauser, P.D. Home, R. Worth, S. Sailer, A.B. Kurtz, M. Christy, A prospective study of the immunogenicity of porcine insulin in HLA-typed new insulin-treated diabetics, Diabetes Res 2, (1985) 221-224.

2. J. Baselga, J. Mendelsohn, The epidermal growth factor receptor as a target for therapy in breast carcinoma, Breast Cancer Res and Treatment 29 (1994) 127-138.

3. P. Chen, M. Mrkobrada, K.A. Vallis, R.M. Reilly, Comparative cytotoxicity of Auger electron therapy, γ-radiation and chemotherapeutics against EGFR-positive breast cancer cells, Proc Amer Assoc Cancer Res 41 (2000) 706 (Abstract 4490).

4. H.S. Earp, T.L. Dawson, X. Li, H. Yu, Heterodimerization and functional interaction between EGF receptor family members: A new signaling paradigm with implications for breast cancer research, Breast Cancer Res Treat 35 (1995) 115-132.

5. J. Filmus, M.N. Pollak, R. Cailleau, R.N. Buick, A human breast cancer cell line with a high number of epidermal growth factor (EGF) receptors, has an amplified EGF receptor gene and is growth inhibited by EGF, Biochem Biophys Res Commun 128 (1985) 898-905.

6. S.L. Fitzpatrick, M.P. LaChance, G.S. Schultz, Characterization of epidermal growth factor receptor and action on human breast cancer cells in culture, Cancer Res. 44 (1984) 3442-3447.

7. K. Fujimori, D.G. Covell, J.E. Fletcher, J.N. Weinstein, A modeling analysis of monoclonal antibody percolation through tumors: a binding site barrier, J Nucl Med 31 (1990) 1191-1198.

8. L. Gedda, P. Olsson, J. Ponten, J. Carlsson, J, Development and in vitro studies of epidermal growth factor-dextran conjugated for boron neutron capture therapy, Bioconjugate Chem 7 (1996) 584-591.

9. Genbank. www.ncbi.nlm.nih.gov Human Serum Albumin. (Accession No. V00495). Bethesda, MD, National Center for Biotechnology Information, 3-1-2000.

10. A. Gijsens, L. Missiaen, W. Merlevede, P. de Witte, Epidermal growth factor-mediated targeting of chlorin e_6 selectively potentiates its photodynamic activity, Cancer Res 60 (2000) 2197-2202.

11. D.J. Hnatowich, R.L. Childs, D. Lanteigne, A. Najafi, The preparation of DTPA-coupled antibodies radiolabeled with metallic radionuclides: an improved method, J Immunol Meth 65 (1983) 147-157.

12. U. Hommel, T.S. Harvey, P.C. Driscoll, I.D. Campbell, Human epidermal growth factor: high resolution solution structure and comparison with human transforming growth factor α, J Mol Biol 227 (1992) 271-282.

13. R.K. Jain, Physiological barriers to delivery of monoclonal antibodies and other macromolecules in tumors, Cancer Res. 50 (1990) 814s-819s.

14. J.G.M. Klijn, P.M.J. Berns, P.I.M. Schmitz, J.A. Foekens. The clinical significance of epidermal growth factor receptor (EGF-R) in human breast cancer: a review on 5232 patients, Endocr Rev 13 (1992) 3-17.

15. H.J. Motulsky, P. Stannard, R. Neubig, Prism. Ver 2.0. San Diego, GraphPad Software Inc. 1995.

16. E.M. Rakowicz-Szulczynska, U. Rodeck, M. Herlyn, H. Koprowski, H. Chromatin binding of epidermal growth factor, nerve growth factor, and platelet-derived growth factor in cells bearing the appropriate surface receptors, Proc Natl Acad Sci 83 (1986) 3728-3732.

17. R.M. Reilly, J. Sandhu, T. Alvarez-Diez, S. Gallinger, J. Kirsh, H.S. Stern, Problems of delivery of monoclonal antibodies: Pharmaceutical and pharmacokinetic solutions, Clin Pharmacokinet 28 (1995) 126-142.

18. R.M. Reilly, R. Kiarash, R. Cameron, N. Porlier, J. Sandhu, R.P. Hill, K. Vallis, A. Hendler, J. Gariépy, ^{111}In-labeled EGF is selectively radiotoxic to human breast cancer cells overexpressing EGFR, J Nucl Med 41 (2000) 429-438.

19. R.M. Reilly, R. Kiarash, J. Sandhu, Y.W. Lee, R. Cameron, A. Hendler, K. Vallis, J. Gariépy, (2000) A comparison of EGF and mAb 528 labeled with ^{111}In for imaging human breast cancer, J Nucl Med 41 (2000) 903-911.

20. G. Stehle, H. Sinn, A. Wunder, H.H. Schrenk, J.C.M. Stewart, G. Hartung, W. Maier-Borst, D.L. Heene, Plasma protein (albumin) catabolism by the tumor itself-implications for tumor metabolism and the genesis of cachexia, Crit Rev Oncol Hematol 26 (1997) 77-100.

21. T. Yokota, D.E. Milenic, M. Whitlow, J. Schlom, Rapid tumor penetration of a single-chain Fv and comparison with other immunoglobulin forms, Cancer Res 52 (1992) 3402-3408.

Legends for Figures

Fig. 1. Construction of HSA-hEGF bioconjugate. A. HSA was thiolated by reaction with 2-iminothiolane. B. hEGF was derivatized with maleimide by reaction with sulfo-SMCC. C. Thiolated HSA was reacted with maleimide-derivatized hEGF.

Fig. 2. SDS-PAGE analysis of HSA-hEGF on a non-reducing 4-20% Tris HCl gradient mini-gel stained with Coomassie brilliant blue. Lane 1: Molecular weight standards. Lane 2: hEGF. Lane 3: HSA. Lane 4: HSA-hEGF.

Fig. 3. Size-exclusion HPLC of HSA-hEGF on a Progel TSK swxl G2000 column eluted with 100 mM KH_2PO_4/100 mM Na_2SO_4 pH 7.0 at a flow rate of 1 mL/min with UV detection at 280 nm.

Fig. 4. Fluorescence microscopy of MDA-MB-468 human breast cancer cells incubated with A. fluorescein-derivatized HSA-hEGF or B. DAPI.

Fig. 5. A. Effect of ^{111}In-DTPA-HSA-hEGF or ^{111}In-DTPA-hEGF on the growth of MDA-MB-468 human breast cancer cells overexpressing EGFR (1-2 $\times$ 10^6 receptors/cell) or B. Effect of ^{111}In-DTPA-HSA-hEGF or ^{111}In-DTPA-hEGF on the growth of MCF-7 cells with a 100-fold lower level of EGFR expression (1 $\times$ 10^4 receptors/cell). C. Effect of ^{111}In-DTPA-hEGF or unlabeled hEGF on the growth of MDA-MB-468 human breast cancer cells.

TABLE 1. Receptor-Binding Properties of ^{111}In-DTPA-HSA-hEGF Multiply Substituted with DTPA.

Molar Ratio (cDTPAA: HSA-hEGF)	**DTPA Substitution Level (moles DTPA/mole HSA-hEGF)**	**K_a (L/mole × 10^7)**	**B_{max} (sites/cell × 10^6)**
10:1	1.6 ± 0.6	5.1 ± 1.3	2.0 ± 0.6
50:1	13.5 ± 2.4	4.7 ± 1.3	1.6 ± 0.1
100:1	22.7 ± 5.0	3.4 ± 0.9	1.4 ± 0.1

DTPA substitution level and receptor-binding parameters are expressed as mean ± s.e.m. of 3-6 experiments. There were no significant differences (t-test, $p<0.05$) in K_a or B_{max} values between any of the bioconjugates.

Figure 1

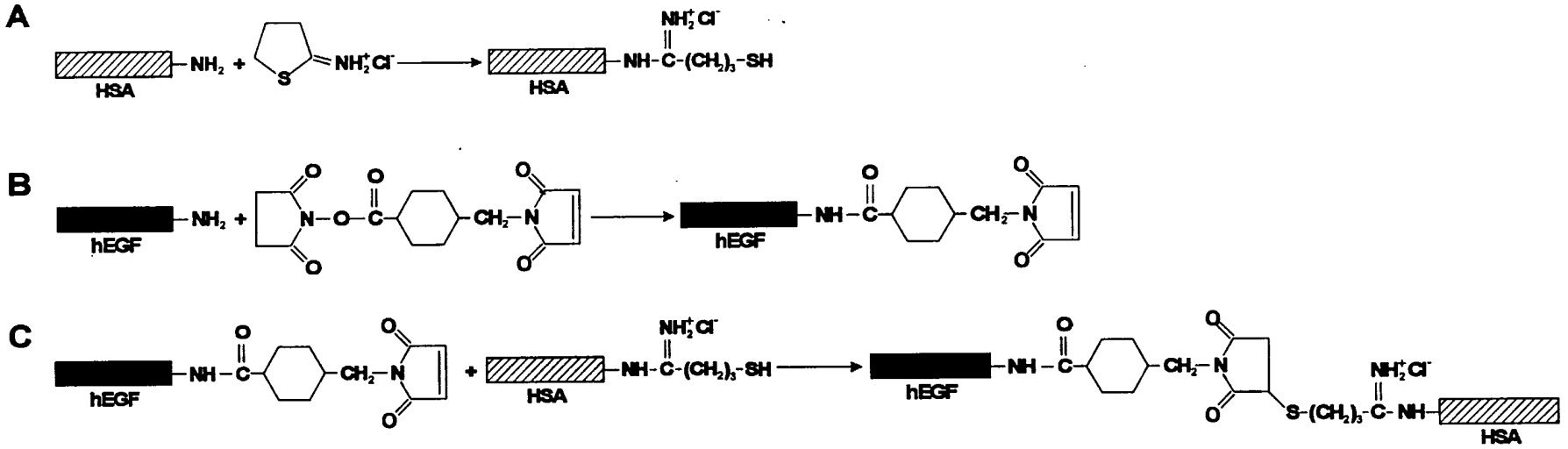

A
HSA
NH2
NH2+Cl-
S
HSA
NH-C-(CH2)3-SH
B
hEGF
NH2
N-O-C
CH2-N
hEGF
NH-C
CH2-N
C
hEGF
NH-C
CH2-N
HSA
NH-C-(CH2)3-SH
hEGF
NH-C
CH2-N
S-(CH2)3-C-NH
HSA

Figure 2

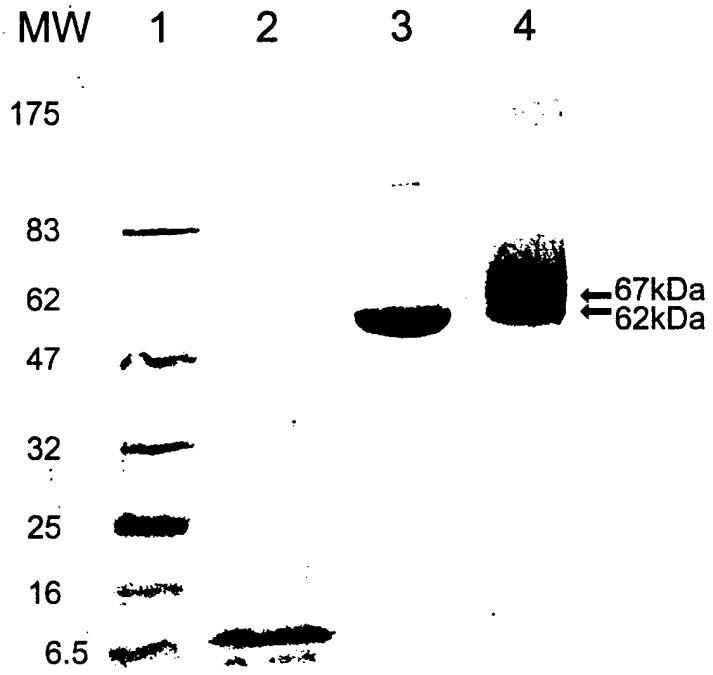


Figure 3

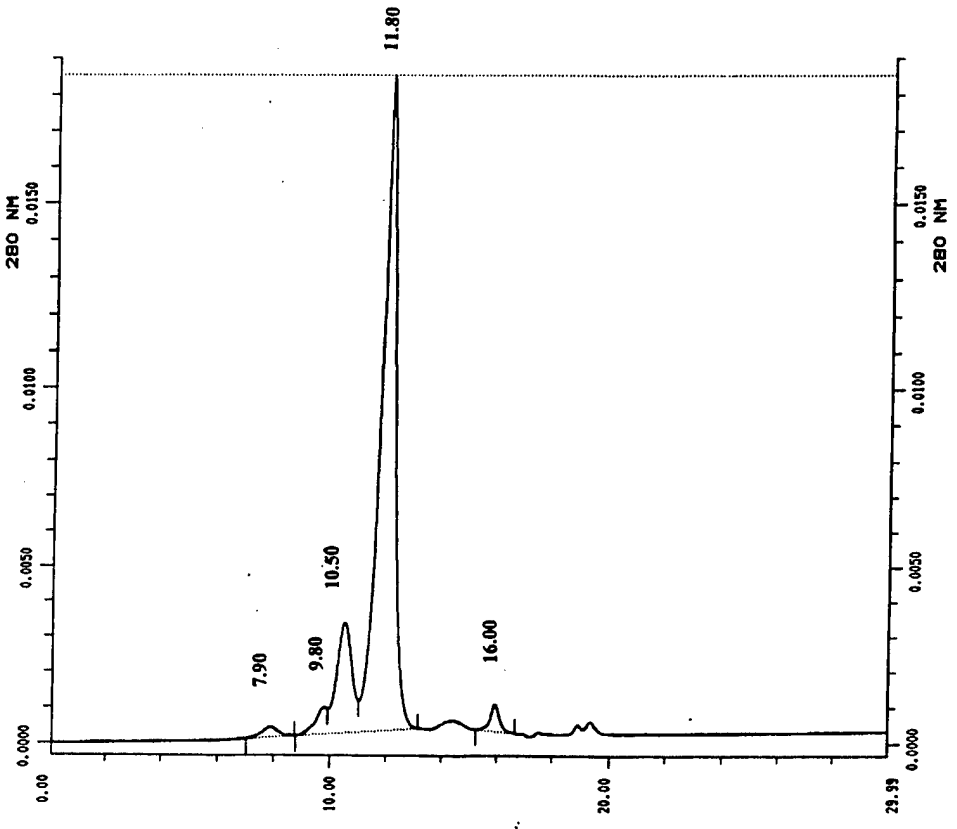

11.80
7.90
9.80
10.50
16.00
280 NM
0.0150
0.0100
0.0050
0.0000
0.00
10.00
20.00
29.99

Figure 4

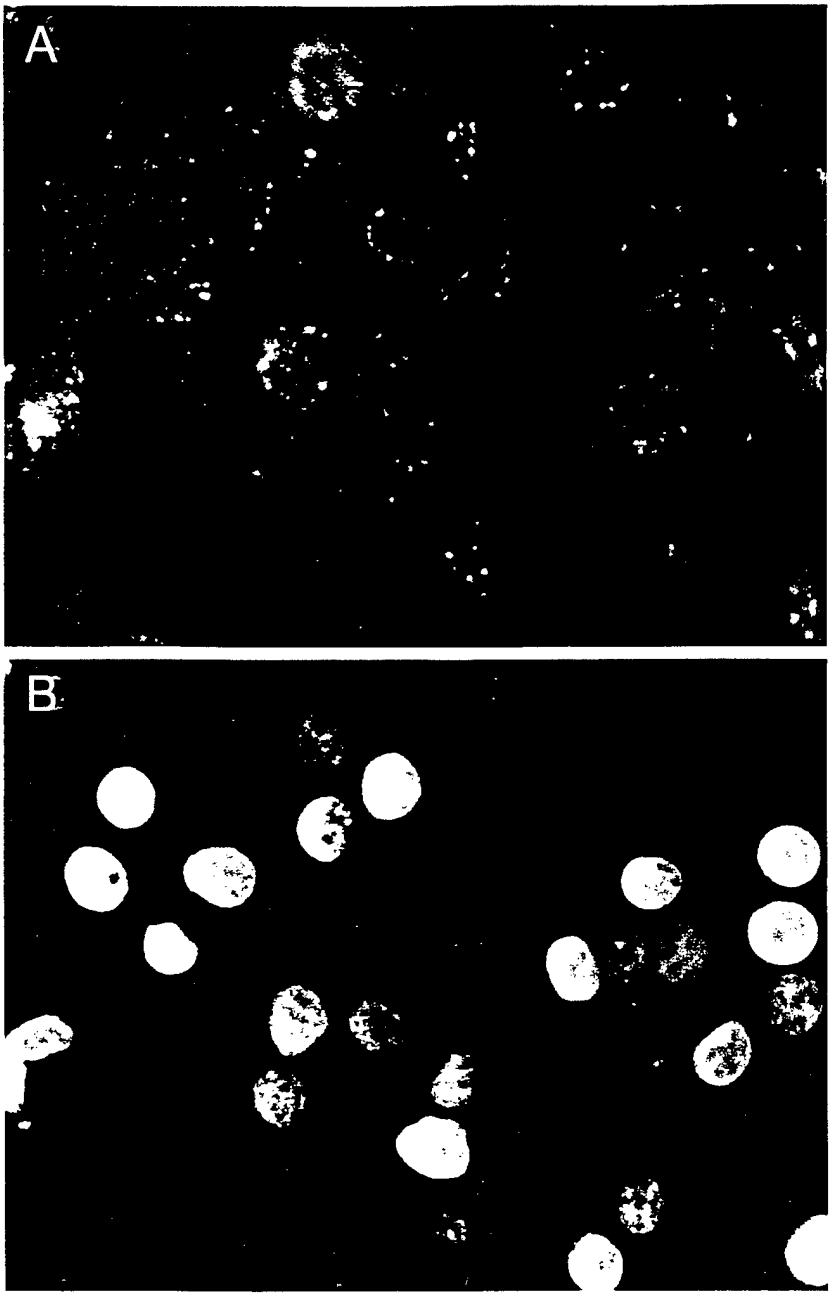
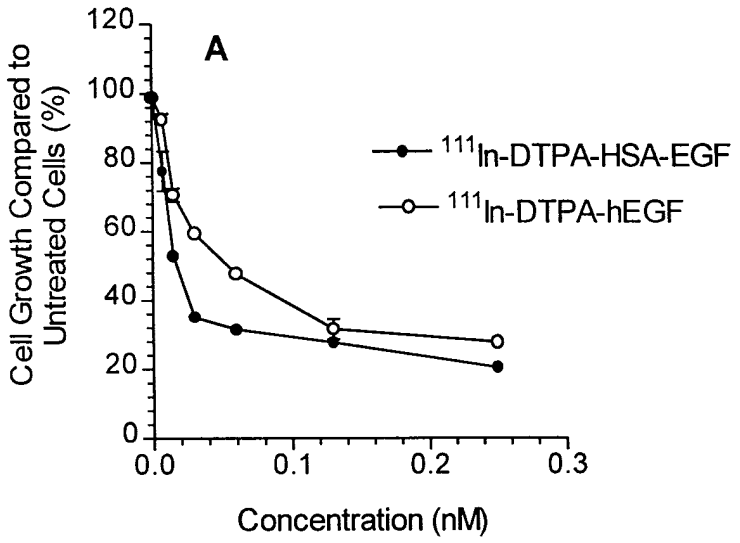
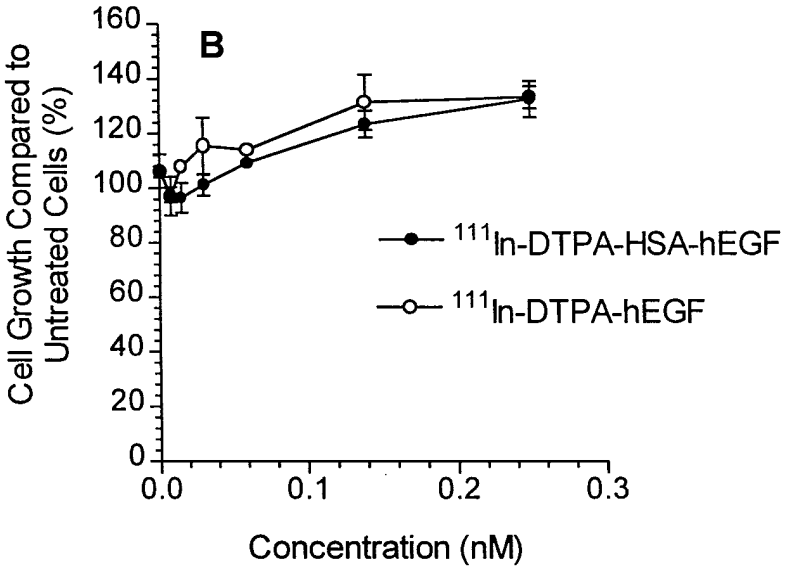
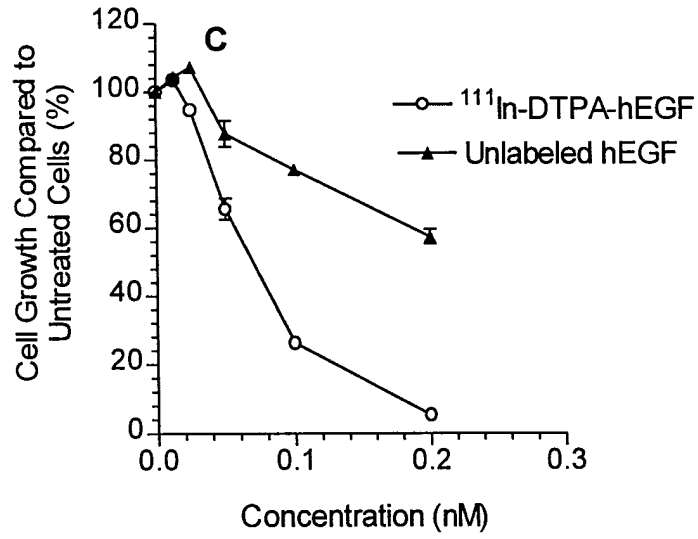


Figure 5


A
Cell Growth Compared to Untreated Cells (%)
120
100
80
60
40
20
0
0.0
0.1
0.2
0.3
Concentration (nM)
111In-DTPA-HSA-EGF
111In-DTPA-hEGF


B
Cell Growth Compared to Untreated Cells (%)
160
140
120
100
80
60
40
20
0
0.0
0.1
0.2
0.3
Concentration (nM)
111In-DTPA-HSA-hEGF
111In-DTPA-hEGF


C
Cell Growth Compared to Untreated Cells (%)
120
100
80
60
40
20
0
0.0
0.1
0.2
0.3
Concentration (nM)
111In-DTPA-hEGF
Unlabeled hEGF

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THE ANTI-TUMOR EFFECT OF THE AUGER ELECTRON-EMITTING RADIOPHARMACEUTICAL, ^{111}In-hEGF AGAINST MDA-MB-468 HUMAN BREAST CANCER XENOGRAFTS IS TUMOR-SIZE DEPENDENT.

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University Health Network, Toronto, Canada and Samuel Lunenfeld Research Institute, Toronto, Canada

Rationale: Auger electron-emitting radiopharmaceuticals (AER) exert cytotoxic effects on single cells. Small tumors may be more sensitive to AER due to greater targeting of individual cancer cells combined with higher tumor uptake (% i.d./g). **Objectives**: Our objective was to determine if ^{111}In-human epidermal growth factor (hEGF) inhibited the growth of EGFR-overexpressing MDA-MB-468 breast cancer xenografts in nude mice and if anti-tumor effects were tumor size-dependent. **Methods**: Groups of 5 mice with small (4 mm^3) or large (21 mm^3) s.c. MDA-MB-468 tumors were administered 6 weekly s.c. injections of ^{111}In-hEGF (total 0.75-2.5 mCi, 5-17 μg) or hEGF (17 μg). One group received doxorubicin (5 mg/kg × 2 days). Tumor diameter (d) was measured twice/wk for 7 wks and tumor volume calculated. Normal tissue toxicity was evaluated by measuring body weight, WBC and platelet counts and pathological examination of tissues by electron microscopy (EM). **Results**: Small tumors exhibited almost complete growth arrest after 2.5 mCi of ^{111}In-hEGF (6.5 ± 4.5 mm^3 vs. 34.1 ± 13.7 mm^3 for untreated mice). hEGF had no effect on tumor growth (35.7 ± 21.5 mm^3). Large tumors exhibited only 30-35% decreased growth even at higher amounts of ^{111}In-hEGF (1.5 or 2.5 mCi). Doxorubicin was effective against MDA-MB-468 tumors but caused death in 2/5 mice. No deaths occurred with ^{111}In-hEGF and no change in body weight was noted (25.0 ± 0.6 g vs. 23.6 ± 0.5 g for untreated mice). Doxorubicin caused a 20% loss in body weight (19.6 ± 2.2 g). WBC and platelets remained in the normal range except for doxorubicin which caused 4-fold decreased platelets. No normal tissue toxicity was observed by EM for ^{111}In-hEGF but doxorubicin caused cardiotoxicity. **Conclusions**: ^{111}In-hEGF is a promising new radiotherapeutic agent for EGFR-overexpressing breast cancer which does not appear to be associated with significant normal tissue toxicity. The anti-tumor effects of the radiopharmaceutical are tumor-size dependent with small tumors exhibiting the greatest response. Supported by U.S. Army Breast Cancer Research Program Grant DAMD17-98-1-8171.